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THE ROLE OF THE ADRENAL CORTEX IN THE NORMAL ADAPTATION TO DIETARY SODIUM DEPRIVATION*

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ABSTRACT

During short periods of rigid dietary sodium deprivation, there was no significant change in the urinary output of total 17-hydroxycorticoids in a normal subject and in a patient with intact adrenals given a constant quantity of cortisone orally. The urinary excretion of cortisol and cortisone was also unaffected under these circumstances. The administration of a constant dose of prednisone or deoxycorticosterone to patients with Addison's disease did not permit normal adaptation to acute dietary sodium deprivation, although the latter was much more effective than the former.

These observations tend to emphasize the physiologic importance of a rise in the urinary excretion of aldosterone in the adaptation to sodium restriction, and it would appear that the rise in urinary levels of aldosterone during a low

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sodium diet is a reflection of an increased secretion of this hormone rather than a result of alteration in its intermediary metabolism.

THE inability of the body to retain sodium adequately is a prominent feature of adrenocortical insufficiency (1, 2). This defect can be overcome by the administration of large amounts of sodium chloride (3) or remedied by the injection of adrenocortical extract (4). With the discovery by Simpson, Tait and co-workers, of aldosterone in adrenal extracts (5) and in adrenal vein blood (6), the problem of adrenal participation in the reaction of the organism to sodium deprivation can be approached more definitively. Since adrenocortical hormones other than aldosterone are also capable of producing sodium retention, the question arises as to the extent to which these substances may contribute to the adaptation of the human organism to sodium deprivation.

The problems that will be considered are the following: 1) Is there any evidence for an increase in the secretion of cortisol, quantitatively the major corticosteroid present in human peripheral blood (7, 8), during exposure to sodium deprivation? 2) If not, is the urinary excretion of cortisol modified during sodium depletion? 3) Conversely, does the administration of a constant quantity of a steroid resembling cortisol in its physiologic properties, enable the organism to withstand sodium deprivation? In other words, can such a steroid exert on the mechanisms for sodium conservation a "permissive action," in the sense used by Ingle (9)?

It will be shown that these questions can be answered in the negative. The role played by aldosterone in the regulation of electrolyte balance will be described in more detail in the accompanying paper (10).

METHODS

The studies were performed on the Metabolic Ward of the Peter Bent Brigham Hospital on subjects maintained on a constant diet. The composition of the diet was calculated from the tables of Bowes and Church (11). Body weight was recorded daily at the beginning of the metabolic day (8 A.M.). Collections of saliva for sodium and potassium determinations were obtained at the same time, using the procedure of Frawley and Thorn (12). The total amount of urine passed during the twenty-four hours was measured and stored at 4° C. Urinary creatinine was determined according to the method of Bonsnes and Taussky (13); urinary sodium concentration was measured using the flame photometer, with lithium as the internal standard.

Urinary 17-hydroxycorticoids were measured by a modification (14) of the method of Reddy (15). Urinary aldosterone was measured by colorimetry after chromatographic isolation (16). Essentially the same procedure was used for measuring urinary cortisol and cortisone. The latter was isolated from the Bush "C" chromatogram; after elution it was measured by the reaction of Porter and Silber (17), or, in one study, by that of Gornall and MacDonald (18). Cortisol was isolated and eluted from the Zaffaroni chromatogram, rechromatographed on the Bush "B5" system (19) and measured by the blue tetrazolium reaction (20), or in one study, by the reaction of Gornall and MacDonald (18).

Tests were performed on pools of aldosterone, cortisol and cortisone, so obtained to assess the chemical purity of these steroids. Aldosterone was found to be contaminated sometimes by substances separable from it after additional chromatography on the "E_2B" system of Eberlein and Bongiovanni (21). The amounts of the contaminating material, less polar than aldosterone on this system, were small; furthermore, this material did *not* reduce blue tetrazolium immediately, and therefore did not interfere with the final quantitative estimation of aldosterone. It is probably indentical with the mixture of compounds III and IV described by Nowaczynski *et al.* (22). Some samples of cortisone were likewise impure after elution from the Bush "C" paper chromatogram. Under ultraviolet light, the contaminating substance was found to be less polar than cortisone when chromatographed on the "E_2B" system of Eberlein and Bongiovanni (21), or on the "B_2" system of Bush (19) after acetylation. This material did not cause immediate reduction of blue tetrazolium. No further attempts were made to characterize it. The behavior of cortisol isolated from urine by the method used, was identical in both free and acetylated forms with that of appropriate reference standards on several paper chromatographic systems, and no contaminating material could be detected.

RESULTS

I. *Steroid excretion during sodium restriction*

A. Three separate studies of dietary deprivation of sodium were made on a normal subject (J.C.) who was ambulant throughout. In each study the daily sodium intake was approximately 200 mEq. (his normal intake) for two days, then was reduced to exactly 9 mEq. for six days, and then restored to approximately 200 mEq. for two days. The excretion of 17-hydroxycorticoids, cortisol and cortisone was measured before, during and after 3 separate six-day periods of sodium restriction, as shown in Figure 1. During the twelve days of high sodium intake the mean level of excretion of total 17-hydroxycorticoids was 9.5 mg. per twenty-four hours (S.D. $\pm$2.0 mg.). During the eighteen days of sodium restriction it was 7.9 mg. per twenty-four hours (S.D. $\pm$2.0 mg.). This difference is not significant ($P > 0.05$), and both sets of values are within the range for healthy young adult males (23). During the twelve days of high sodium intake, the mean excretion of cortisol was 10.0 μg., and of cortisone 9.5 μg. per twenty-four hours. During the eighteen days of sodium restriction, the excretion of cortisol amounted to 9.5 μg., and of cortisone to 11.0 μg. per twenty-four hours. Thus, no differences in excretion were observed for either steroid during high and low sodium intakes.

B. A 71-year-old man with gouty arthritis (A.L.) who had been maintained with 50 mg. of cortisone daily for five years was likewise subjected to sodium restriction. Assuming that the exogenously administered corticoid would abolish most if not all of his endogenous cortisol production (24), this study could provide information as to the proportion of cortisone excreted in the urine as such, of that converted to and excreted as cortisol,

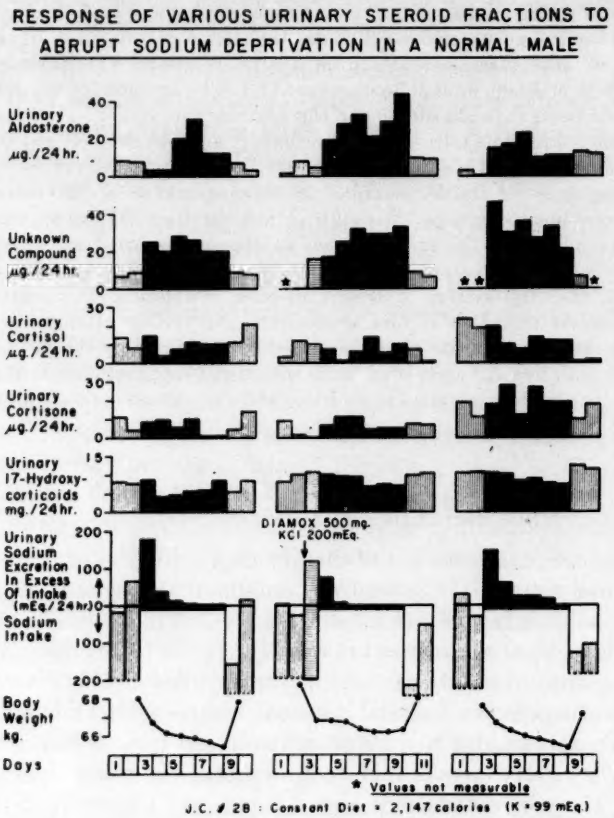
RESPONSE OF VARIOUS URINARY STEROID FRACTIONS TO
ABRUPT SODIUM DEPRIVATION IN A NORMAL MALE
Urinary Aldosterone µg./24 hr.
Unknown Compound µg./24 hr.
Urinary Cortisol µg./24 hr.
Urinary Cortisone µg./24 hr.
Urinary 17-Hydroxy-corticoids mg./24 hr.
DIAMOX 500 mg.
KCl 200 mEq.
Urinary Sodium Excretion In Excess Of Intake (mEq./24 hr.)
Sodium Intake
Body Weight kg.
Days
* Values not measurable
J.C. # 28 : Constant Diet = 2,147 calories (K = 99 mEq)

FIG. 1. In this study, urinary cortisol was measured by the blue tetrazolium reaction, and urinary cortisone by the reaction of Porter and Silber. For the unknown compound, see text.

and that converted to and excreted as metabolites possessing an unaltered side-chain measurable in the urine as 17-hydroxycorticoids.

Studies on this patient revealed that the amount of 17-hydroxycorticoids excreted was little changed during the period of sodium restriction (Fig. 2). With a sodium intake of 140 mEq., the mean excretion of total 17-hydroxycorticoids over four days was 24.0 mg. per twenty-four hours; during the six-day period with a sodium intake of 20 mEq. daily, it was 26.8 mg. per twenty-four hours. The excretion of cortisol also did not change significantly during the period of sodium restriction (Fig. 2); during the control days the mean excretion was 3 µg., and during the period of sodium restriction 2 µg. per twenty-four hours. The excretion of corti-

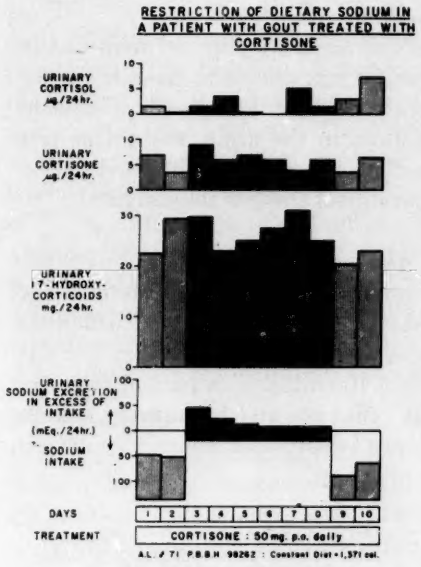
RESTRICTION OF DIETARY SODIUM IN A PATIENT WITH GOUT TREATED WITH CORTISONE
URINARY CORTISOL µg./24hr.
URINARY CORTISONE µg./24hr.
URINARY 17-HYDROXY-CORTICOIDS mg./24hr.
URINARY SODIUM EXCRETION IN EXCESS OF INTAKE (mEq./24hr.)
SODIUM INTAKE
DAYS
TREATMENT
CORTISONE : 50 mg. p.o. daily

FIG. 2. Urinary cortisol and cortisone were measured by means of the reaction of Gornall and MacDonald.

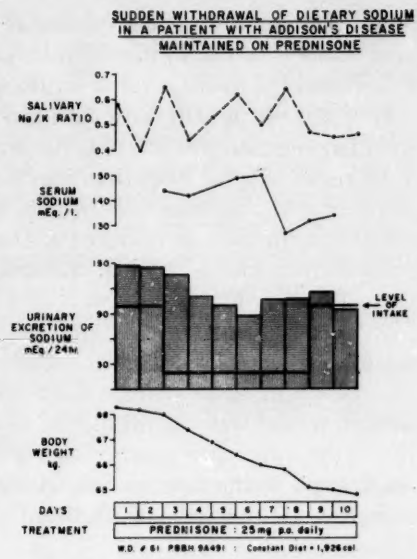
SUDDEN WITHDRAWAL OF DIETARY SODIUM IN A PATIENT WITH ADDISON'S DISEASE MAINTAINED ON PREDNISONE
SALIVARY Na/K RATIO
SERUM SODIUM mEq./l.
URINARY EXCRETION OF SODIUM mEq./24hr.
LEVEL OF INTAKE
BODY WEIGHT kg.
DAYS
TREATMENT
PREDNISONE : 25 mg. p.o. daily

FIG. 3. This presentation of the sodium "balance" in this patient is aimed at outlining the almost unaltered rate of excretion of sodium despite reduction in dietary intake of the electrolyte, in body weight and in serum sodium concentration.

sone amounted to 5 µg. during normal sodium intake and 6 µg. per twenty-four hours during sodium restriction.

II. *Dietary deprivation of sodium in patients with Addison's disease maintained with a constant dosage of steroids*

Because of the failure to demonstrate gross changes in the production and metabolism of endogenous cortisol, and in the fate of exogenously administered cortisone during sodium restriction, studies were performed on patients with Addison's disease to determine whether or not sodium retention occurred when these patients were subjected to sodium restriction while receiving a constant daily dose of 1) a type of adrenal steroid which possessed minimal sodium-retaining properties, or 2) one with marked sodium-retaining properties.

A. A 61-year-old man with Addison's disease (W.D.) was given a constant dose (25 mg.) of prednisone daily during a reduction in sodium intake from 100 mEq. per day to 20 mEq. per day for six days, after which the sodium intake was restored to a level of 100 mEq./day. The inability to

retain sodium during the period of sodium restriction while receiving prednisone is shown in Figure 3. Urinary sodium loss was accompanied by a profound fall in the level of serum sodium.

In a second patient with Addison's disease, a 61-year-old man (J.C.), a similar regimen was started. However, he was unable to tolerate the sodium restriction for more than four days when maintained with prednisone alone (Fig. 4), although the loss of sodium in the urine was not as pronounced as in the first patient (W.D.). His urinary excretion of sodium decreased from 100 to 72 mEq. on the first day of the low sodium intake but thereafter was little changed.

B. A third patient with Addison's disease (J.S.), 53 years of age, was maintained with 10 mg. of deoxycorticosterone acetate administered intramuscularly in oil as a single daily dose. After two days during which the sodium intake was 100 mEq., the intake was reduced to 20 mEq. per day for six days and then restored to a level of 100 mEq. This patient tolerated six days of sodium restriction without difficulty and his urinary sodium excretion fell to a level of 30–40 mEq. per twenty-four hours, as shown in Figure 5.

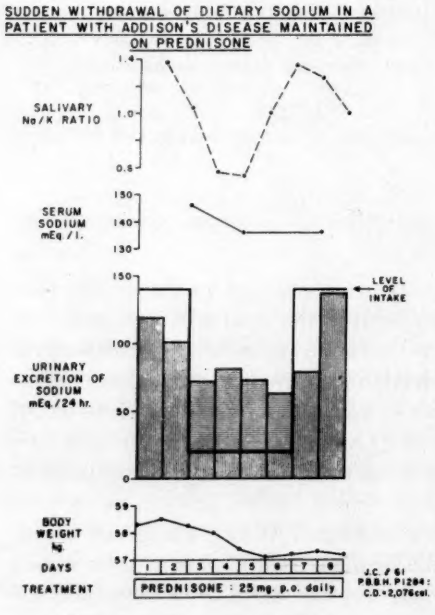
SUDDEN WITHDRAWAL OF DIETARY SODIUM IN A PATIENT WITH ADDISON'S DISEASE MAINTAINED ON PREDNISONE
SALIVARY Na/K RATIO
SERUM SODIUM mEq/l.
URINARY EXCRETION OF SODIUM mEq./24 hr.
LEVEL OF INTAKE
BODY WEIGHT kg.
DAYS
TREATMENT
PREDNISONE : 25 mg. p.o. daily
J.C. / 61
P.B.S.H. P1284
C.D. = 2,076 col.

FIG. 4. See legend for Figure 3.

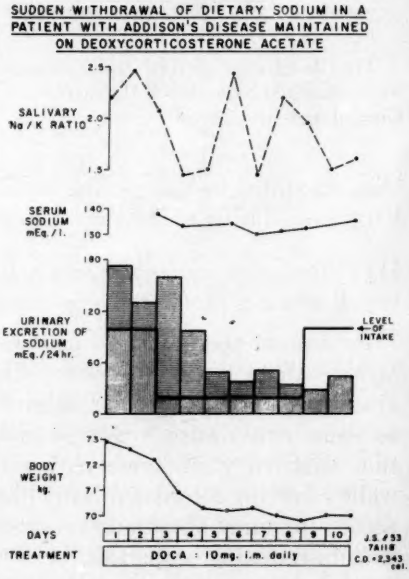
SUDDEN WITHDRAWAL OF DIETARY SODIUM IN A PATIENT WITH ADDISON'S DISEASE MAINTAINED ON DEOXYCORTICOSTERONE ACETATE
SALIVARY Na/K RATIO
SERUM SODIUM mEq/l.
URINARY EXCRETION OF SODIUM mEq./24 hr.
LEVEL OF INTAKE
BODY WEIGHT kg.
DAYS
TREATMENT
DOCA : 10 mg. i.m. daily
J.S. / 53
C.D. = 2,343 col.

FIG. 5. This presentation of sodium "balance" is thought to illustrate better the lack of normal adaptation to dietary sodium deprivation.

C. Differences in the salivary Na/K ratios of Patients W.D. and J.S., both suffering from Addison's disease, are worthy of note. The salivary Na/K ratio of Patient J.S. when given 10 mg. of deoxycorticosterone daily, was 2 to 4 times higher than that of Patient W.D. who was receiving prednisone. The difference between these ratios is almost entirely due to the differences in sodium concentration, that of potassium being almost the same in the 2 patients. These observations underline the unreliability of random determinations in salivary Na/K ratios as reflecting quantitatively the activity of sodium-retaining hormones administered to human subjects, although a reduction in this ratio has been observed regularly when serial determinations are performed during continued administration of aldosterone in man.

Furthermore, the progressive fall in the salivary Na/K ratio during the exposure of normal subjects to a low sodium intake (10) did not occur in these 2 patients with Addison's disease.

III. *Rise in the urinary excretion of aldosterone as a result of salt deprivation*

The lack of adequate adjustment of the sodium excretion to drastic reductions in dietary intake of salt while constant quantities of steroids were administered to patients with Addison's disease, emphasizes the importance of the rise in urinary aldosterone values observed characteristically in normal subjects under such circumstances. The significance of this rise, as shown by the elevated levels of the hormone in urine (Fig. 1), will be discussed separately (10).

Also in Subject J.C., the excretion of an unknown compound described previously (16) was measured in urine by means of the blue tetrazolium reaction performed in test tubes (20). The structure of this substance is unknown to date. Its origin is possibly exogenous, since studies in which the diet was constant for periods of five weeks, except for large variations in the intake of salt, indicated that the material was excreted in constant amounts throughout the study period.

DISCUSSION

The present findings indicate that a constant dosage of prednisone, a steroid with weak sodium-retaining properties, does not enable the organism in the absence of the adrenal glands to respond by renal retention of sodium when salt is suddenly withdrawn from the diet. On the other hand, there was a progressive decrease in the excretion of sodium in a patient with Addison's disease given a constant daily dose of deoxycorticosterone acetate as the only maintenance therapy during sodium deprivation. However, with the quantity of hormone employed, sodium excretion did not fall to the level attained by normal subjects deprived of sodium (10).

Van't Hoff (25) and Lipsett and Pearson (26) both noted a progressive fall in urinary sodium excretion when the sodium intake was restricted in adrenalectomized patients receiving constant daily amounts of cortisone; in these cases a persistent negative sodium balance resulted rapidly in circulatory collapse.

Because of the quantitative relationship observed between the degree of urinary sodium retention and the amount of cortisol or cortisone administered (27), the possibility arises that the adrenal secretion of cortisol is increased under conditions of low sodium intake. The balance studies of Leaf and Couter (28) on normal subjects exposed to abrupt withdrawal of dietary sodium revealed an increased excretion of nitrogen during the period of adaptation to such a diet; this led the authors to conclude that these experimental conditions stimulated adrenocortical activity. Except for Renwick *et al.* (29), who reported a small rise of urinary corticoids in man during sodium deprivation, investigators (30–33) have sought unsuccessfully for evidence in support of this possibility. Using a method of measurement resembling closely that employed in the present studies, Luetscher and Axelrad (34), and Muller *et al.* (35), also failed to demonstrate any change in the excretion of 17-hydroxycorticoids in normal subjects given a low-sodium diet. The results of the present investigations are likewise negative in that respect, which suggests that in so far as the renal excretion of 17-hydroxycorticoids in man reflects adrenal secretion of cortisol, the latter is unaffected by changes in the salt content of the diet. Measurements of cortisol in the adrenal vein blood of dogs confirm this suggestion, as they showed no increase during sodium deprivation (36).

The discrepancy between some of the observations referred to could be due, in part at least, to modification in steroid metabolism during sodium deprivation. Such a modification could hypothetically result in the increased effectiveness of circulating cortisol, whereas its production by the adrenal cortex would be unaffected. This would account for Leaf and Couter's observations already mentioned, as well as the finding of Lipsett and Pearson (37), who observed in adrenalectomized patients the same phenomenon of a transient negative nitrogen balance when salt was withdrawn from the diet while the patient was receiving a constant amount of cortisone throughout the control and experimental periods. No measurement of plasma cortisol was made in the present study, but an index of the metabolism of this hormone was provided by a study of the amount excreted in the urine as biologically active material, namely cortisol and cortisone. Both were present in the urine in similar quantities during the control and experimental periods, in the normal subject as well as in Patient A.L. given a constant dose of cortisone. In the latter there was no indication of any gross deviation in the extent of degradation of exoge-

nous cortisone when he was receiving a low sodium intake, since the 17-hydroxycorticoids were excreted in unchanged amounts throughout the study.

The quantities of cortisol and cortisone found in the urine were very small—a few micrograms a day. The values for cortisol are in satisfactory agreement with those reported by Ayres and co-workers (38) when correction is made for recovery rates (16). Cortisone was reported to be excreted in the urine in quantities of the same order as those of cortisol (39) and the present findings are in agreement with this. The output of large quantities of 17-hydroxycorticoids (20 to 30 mg. per twenty-four hours) contrasts with the minute amounts of cortisone and cortisol (0 to 10 μg. per twenty-four hours) present in the urine of Patient A.L. given 50 mg. of cortisone by mouth daily. The observations of Nelson and Harding (40) and the recent work of Hechter *et al.* (41) point to the surprisingly rapid rate of degradation of corticosteroids by the liver; and ingested cortisone will pass through the liver before its distribution to the systemic circulation. The presence of cortisol in the urine of Patient A.L. could have resulted from the conversion of cortisone to cortisol as has been shown to occur in liver slices (42, 43) and in adrenalectomized patients (44).

It is hoped that these studies on the metabolism of cortisol will serve to validate a basic assumption underlying studies on the *excretion of aldosterone*, namely that an increased level of excretion of the hormone results from an equally increased rate of its secretion by the adrenal cortex. The observations on the urinary excretion of 17-hydroxycorticoids, cortisol and cortisone in the normal subject and in Patient A.L. suggest that the general pathways of corticosteroid metabolism are not significantly altered when the sodium intake is reduced to low levels. This would indicate that the rise in urinary excretion of aldosterone observed under such conditions very likely reflects a rise in the production of the hormone rather than a disturbance in its degradation. Recently, tracer studies with 16-H^3 aldosterone by Ayers and co-workers (45) have demonstrated that the proportion of radioactive aldosterone recovered in the urine after intravenous administration is constant, regardless of the sodium content of the diet.

The present observations serve also to heighten the significance of the increased excretion of aldosterone during sodium deprivation, as there is evidence neither of a change in the secretion of cortisol nor that a constant administration of steroids enables an adequate reduction in urinary excretion of sodium, under such circumstances.

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THE SIGNIFICANCE OF THE SECRETION OF ALDOSTERONE DURING DIETARY SODIUM DEPRIVATION IN NORMAL SUBJECTS*

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The Departments of Medicine, Peter Bent Brigham Hospital and the Harvard Medical School, Boston, Massachusetts

ABSTRACT

The excretion of aldosterone in urine was studied in 11 instances of dietary sodium deprivation in 6 young males. In several instances, there was a lag before the excretion of aldosterone increased. The maximal, as well as the mean, levels of aldosterone excretion attained were highly variable from individual to individual and bore little relationship to the efficiency with which the adaptation to the low-sodium diet took place. In 9 out of 11 cases, the urinary aldosterone fell after a maximum had been reached, although not necessarily to control levels, despite continuing improvement of sodium retention by the kidney. The suddenness of the sodium deprivation seemed to play a role in the extent of the ensuing rise in aldosterone. Resumption of a normal-sodium diet was initially accompanied by efficient sodium retention, although aldosterone excretion in the urine had already decreased to values near or within control levels. These observations suggest the importance of factors other than the amount of aldosterone secreted, in the regulation of sodium excretion by the normal kidney.

THE sudden withdrawal of sodium from the diet of normal subjects results in increased excretion of aldosterone in urine (1–4). This rise is likely to be the reflection of a proportionally increased rate of secretion of the hormone by the adrenal cortex, and to be of significance in the adapta-

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tion of the human organism to this situation for the reasons discussed in the preceding paper (5).

Bartter provided evidence that the increased aldosterone excretion resulting from a low sodium intake is secondary and proportional to the ensuing decrease in volume of the extracellular fluid compartments (1, 2). However, in studies undertaken in this laboratory it has been noted repeatedly that the excretion of aldosterone in the urine increases during sodium deprivation but does not necessarily do so immediately nor does it generally remain at a high level for the duration of the period of sodium restriction. Furthermore, it has been observed that a sodium deficit of given magnitude induces a rise in aldosterone excretion of varying extent according to the subject and to the method whereby this sodium deficit is achieved.

METHODS

Clinical

Eleven studies were performed on 6 adult young males, all normal with the exception of Subject R.A. who suffered from a benign tumor of the spinal cord. The normal men were ambulant during the period of study, and physical exercise likely to produce sweating was avoided. All subjects were weighed at the beginning of the metabolic day (8 A.M.). Urine was collected as 24-hour samples, the volumes of which were measured before storage at 4° C. without preservative.

Unless otherwise stated, the 6 men received a constant diet, the composition of which was calculated from the tables of Bowes and Church (6). The basic diet contained less than 10 mEq. of sodium a day, to which sodium (as sodium chloride) was added for periods and in amounts subsequently stated in detail. Water was allowed without restriction.

Chemical

The following determinations were performed on the urine collections: creatinine, by the method of Bonsnes and Taussky (7); sodium and potassium, by flame photometry; chloride, by the method of Schales and Schales (8); and aldosterone, by the method of Neher and Wettstein (9), as modified by Hernando *et al.* (10). As measured by the latter procedure, the mean daily excretion of aldosterone by normal subjects is 5.0 μg. (S.D. ± 3.0 μg.) and the range is 0 to 15 μg. per twenty-four hours. In 3 subjects, blood was taken for the determination of the serum sodium and potassium concentrations, hematocrit, hemoglobin and serum protein levels. The concentrations of sodium and potassium were measured in saliva collected by the procedure of Frawley and Thorn (11).

RESULTS

1. *Urinary excretion of aldosterone after sudden restriction of dietary sodium intake*

(*a*) A preliminary study was undertaken in 2 normal adult males who received a diet containing less than 10 mEq. of sodium daily for ten days.

The changes in weight and in urinary excretion of sodium and aldosterone with the low-sodium diet are shown in Figures 1 and 2.

In both instances the urinary output of sodium fell below the level of intake—after three days in Subject J.B., and after six days in Subject D.N. There was a sharp fall of body weight in both J.B. and D.N. during the first two days, following which it remained almost constant. In these 2 men there was no rise in the urinary excretion of aldosterone before the second or third day, that is, after the main weight loss had occurred. The excretion of aldosterone then increased, rather unevenly, to reach a maximum on the sixth day in D.N. and on the seventh day in J.B. These peak values were followed by a sudden fall to levels which became stabilized within the normal range during the remainder of the period of low sodium intake. The mean 24-hour aldosterone excretion during the period of sodium restriction was 14.5 μg. in Subject J.B. and 9.5 μg. in Subject D.N.

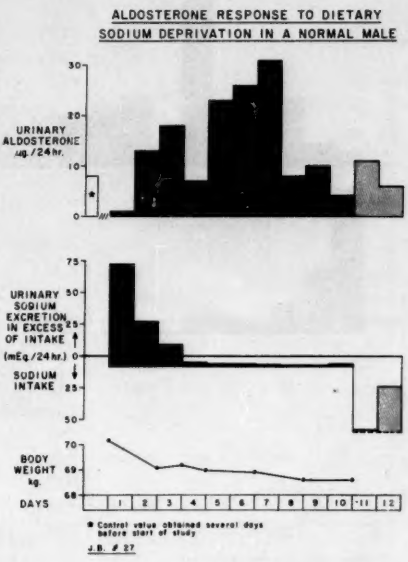
ALDOSTERONE RESPONSE TO DIETARY SODIUM DEPRIVATION IN A NORMAL MALE
URINARY ALDOSTERONE μg./24 hr.
URINARY SODIUM EXCRETION IN EXCESS OF INTAKE (mEq./24 hr.)
SODIUM INTAKE
BODY WEIGHT kg.
DAYS
* Control value obtained several days before start of study
J.B. # 27

FIG. 1. The level of sodium intake on days 11 and 12 is approximate; hence, the dotted line.

In both subjects, resumption of a normal sodium intake resulted in an almost quantitative retention of sodium on the first day. As shown in Figures 1 and 2, this retention was not associated with elevated levels of urinary aldosterone.

(*b*) The pattern of response of urinary aldosterone to dietary sodium deprivation was further studied in 3 normal adult males maintained for twelve days on a constant diet containing 8 mEq. of sodium a day. During days 1 to 3 (Period I) and days 10 to 12 (Period III), 202 mEq. of sodium were taken, as sodium chloride, in addition to the diet; during days 4 to 9 (Period II) the basic diet was taken without supplement. Details of body weight, the excretion of sodium, potassium and aldosterone, the salivary sodium-to-potassium ratios, and blood determinations are given in Table 1 and in Figures 3, 4 and 5.

At the end of the six days of sodium restriction, Subject J.B. had excreted in the urine 168 mEq. of sodium in excess of intake and had lost 1.0 Kg. of body weight. For Subject W.D. the corresponding values were 215

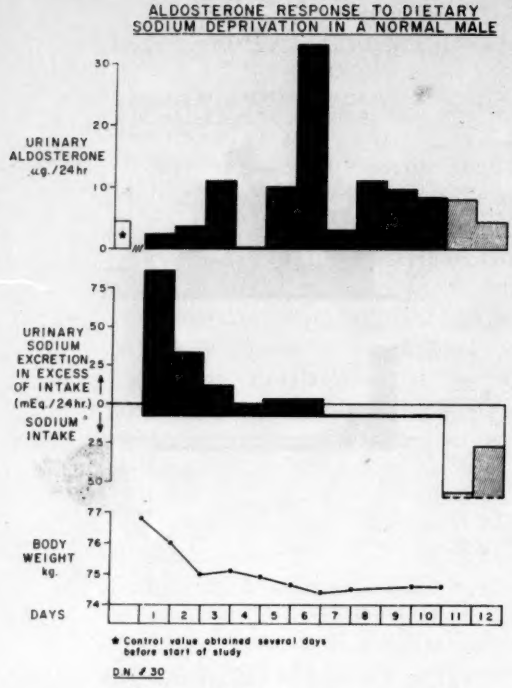
ALDOSTERONE RESPONSE TO DIETARY SODIUM DEPRIVATION IN A NORMAL MALE
URINARY ALDOSTERONE µg./24hr
URINARY SODIUM EXCRETION IN EXCESS OF INTAKE (mEq./24hr.)
SODIUM INTAKE
BODY WEIGHT kg.
DAYS
* Control value obtained several days before start of study
D.N. # 30

FIG. 2. See legend for Figure 1.

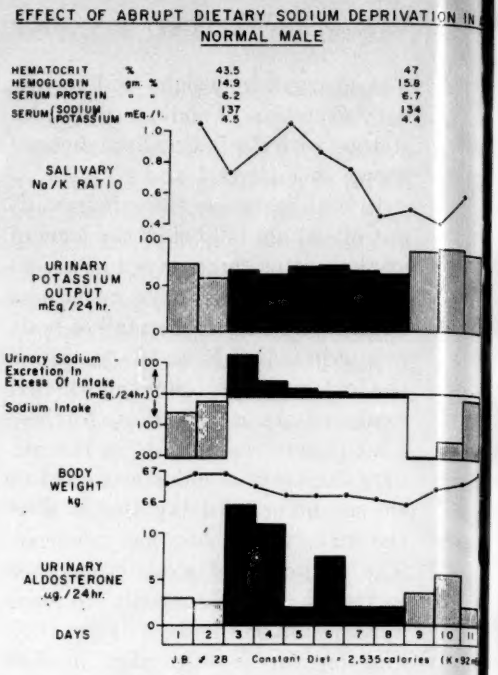
EFFECT OF ABRUPT DIETARY SODIUM DEPRIVATION IN A NORMAL MALE
HEMATOCRIT % 43.5 47
HEMOGLOBIN gm. % 14.9 15.8
SERUM PROTEIN " " 6.2 6.7
SERUM (SODIUM mEq./l. 137 134
SERUM (POTASSIUM 4.5 4.4
SALIVARY Na/K RATIO
URINARY POTASSIUM OUTPUT mEq./24hr.
Urinary Sodium Excretion In Excess Of Intake (mEq./24hr.)
Sodium Intake
BODY WEIGHT kg.
URINARY ALDOSTERONE µg./24hr.
DAYS
J.B. # 28 Constant Diet = 2,535 calories

FIG. 3

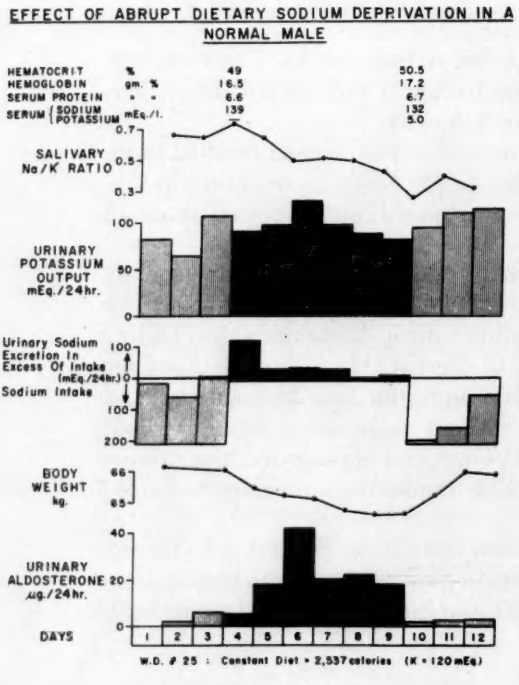
EFFECT OF ABRUPT DIETARY SODIUM DEPRIVATION IN A NORMAL MALE
HEMATOCRIT % 49 50.5
HEMOGLOBIN gm. % 16.5 17.2
SERUM PROTEIN " " 6.6 6.7
SERUM (SODIUM mEq./l. 139 132
SERUM (POTASSIUM 5.0
SALIVARY Na/K RATIO
URINARY POTASSIUM OUTPUT mEq./24hr.
Urinary Sodium Excretion In Excess Of Intake (mEq./24hr.)
Sodium Intake
BODY WEIGHT kg.
URINARY ALDOSTERONE µg./24hr.
DAYS
W.D. # 25 : Constant Diet = 2,537 calories (K = 120 mEq)

FIG. 4

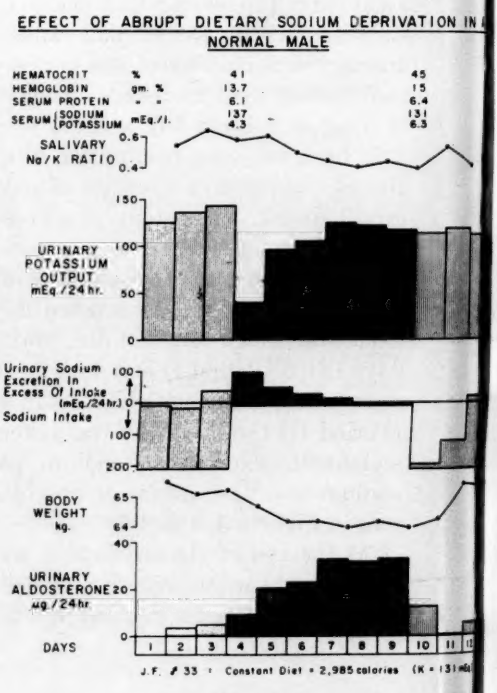
EFFECT OF ABRUPT DIETARY SODIUM DEPRIVATION IN A NORMAL MALE
HEMATOCRIT % 41 45
HEMOGLOBIN gm. % 13.7 15
SERUM PROTEIN " " 6.1 6.4
SERUM (SODIUM mEq./l. 137 131
SERUM (POTASSIUM 4.3 6.3
SALIVARY Na/K RATIO
URINARY POTASSIUM OUTPUT mEq./24hr.
Urinary Sodium Excretion In Excess Of Intake (mEq./24hr.)
Sodium Intake
BODY WEIGHT kg.
URINARY ALDOSTERONE µg./24hr.
DAYS
J.F. # 33 : Constant Diet = 2,985 calories

FIG. 5

TABLE 1. ADAPTATION OF 3 NORMAL MALE SUBJECTS TO LOW SODIUM INTAKE

Period*	Day	Body wt. (Kg.)	Saliva Na (mEq./L)	Saliva K (mEq./L)	Urine values per 24 hrs. Total vol. (ml.)	Creatinine (mg.)	Na (mEq.)	K (mEq.)	Aldosterone (μg.)
		J.B., 28 yrs. Diet: Sodium (mEq./day) 8; Potassium (mEq./day) 92; Calories/day 2535							
I	1								
	2				820	1560	148	72	3.0
	3	66.9	20	19	1040	1400	184	59	2.5
II	4	66.9	13	18	1920	1660	133	68	13.0
	5	66.5	17	19	1920	1520	50	62	11.0
	6	66.2	20	19	1560	1640	25	71	2.0
	7	66.2	18	21	1260	1490	5.7	72	7.5
	8	66.2	15	20	840	1730	1.6	67	2.0
	9	66.1	8.7	19.5	680	1750	1.2	63	2.0
III	10	65.9	11	22.5	660	1770	3.8	87	3.5
	11	66.4	9	22	800	1875	52	90	5.5
	12	66.6	12	20	1060	1910	191	84	2.0
		W.D., 25 yrs. Diet: Sodium (mEq./day) 9; Potassium (mEq./day) 120; Calories/day 2535							
I	1				1540	1850	192	83	—
	2	66.2	11.3	17	1720	2160	148	65	2.0
	3		11	17	2100	2020	212	107	7.0
II	4	66.1	12	16.5	1950	1940	129	89	6.0
	5	65.5	11	17	1410	2280	38	96	18.0
	6	65.3	10	20	1780	2680	39	124	42.0
	7	65.2	10.2	20	1840	2320	37	96	21.0
	8	64.8	10	20	1850	2030	10	87	23.0
	9	64.6	8.3	20	1600	1960	16	83	18.0
III	10	64.7	5.3	20	1420	2050	14	92	2.0
	11	65.3	7.5	18	1000	1840	55	111	3.0
	12	66.1	6.5	20	1420	2030	156	115	3.5

(*Continued on next page*)

* During Periods I and III, sodium chloride (202 mEq. of sodium) was added to the diet, daily.

TABLE 1. (*Continued*)

		J.F., 31 yrs. Diet: Sodium (mEq./day) 8 Potassium (mEq./day) 131 Calories/day 2985							
I	1				1000	2180	200	121	—
	2	65.5	10	18	1250	—	192	136	3.5
	3	65.2	12	19	1400	2320	248	144	5.0
II	4	65.3	11	19	840	1710	106	42	9.0
	5	64.7	11	18	860	1840	57	96	21.0
	6	64.2	10	20	1360	1950	36	106	24.0
	7	64.2	8.9	20	1540	1930	18.6	125	35.0
	8	64.0	8.3	20	2070	1750	8	123	31.0
	9	64.2	10	22	1400	1850	8	119	35.0
III	10	64.0	8.7	22	1300	1880	44	116	13.0
	11	64.3	11	21	840	1820	89	120	0.5
	12	65.4	7.8	19	1660	2180	115	115	6.0

mEq. of sodium and 1.5 Kg. of body weight. In Subject J.F. the values were intermediate—195 mEq. and 1.4 Kg., respectively.

In Subject J.B., the urinary excretion of aldosterone rose immediately, the highest values being observed on the first day of sodium deprivation. In Subject W.D., as in J.F., the rise was not apparent before the second day of low-sodium intake; in W.D. the highest level was attained on the third day of sodium restriction, following which there was stabilization at lower values (around 20 μg. per twenty-four hours) for the last three days of this intake of sodium. In Subject J.F., aldosterone excretion was maximal on the fourth day of sodium deprivation and remained at this level until the conclusion of Period II.

The amounts of aldosterone excreted in the urine of Subject J.B. during the six days of sodium restriction averaged only 6.5 μg. per twenty-four hours—twice the average for the control period. In Subject W.D. the average 24-hour excretion during the low-sodium diet was 21.5 μg., or five times that of the control period, and in Subject J.F. it was 26 μg., or six times that of the control period.

In all 3 subjects, sodium was strongly retained when it was restored to the diet following the period of sodium withdrawal, despite the fact that the urinary excretion of aldosterone promptly returned to normal levels. The urinary excretion of potassium, that had not changed significantly during the period of sodium restriction, tended to rise slightly when sodium was restored to the diet.

In all 3 subjects there was a fall of the salivary sodium/potassium ratio,

due entirely to changes in sodium concentration (Table 1). Although the values during the control period differed widely, the ratios attained at the end of the period of sodium restriction became very similar, approximately 0.4. Noteworthy is the fact that the greatest fall took place in Subject J.B., who excreted the smallest amounts of aldosterone.

2. *Urinary excretion of aldosterone in response to progressive withdrawal of sodium from the diet*

Two of the 3 subjects studied during acute sodium deprivation were maintained for another seventeen days on the same constant diet, containing 8 mEq. of sodium. For the initial two days (Period I) a supplement of 202 mEq. of sodium, as sodium chloride, was added to the diet daily. During the next five days (Period II) the supplement was reduced to 62 mEq. of sodium daily, and to 17 mEq. of sodium daily for the next five days (Period III). For the last five days (Period IV) the diet was taken without additional sodium. In this manner, a stepwise reduction of sodium intake to one third of the level during the previous period was obtained. The data of these studies are summarized in Table 2 and in Figures 6 and 7. Subject J.B. (Fig. 6) excreted 163 mEq. of sodium in the urine in excess of intake during the fifteen days of progressive sodium restriction; this was accompanied by a total weight loss of 1.3 Kg. In J.B., the urinary aldosterone excretion was not correlated closely with the amount of sodium in the urine. In fact, hardly any rise of aldosterone excretion occurred as the result of sodium restriction. When the diet contained 70 mEq. of sodium, the mean aldosterone excretion was 4 μg. per twenty-four hours; it rose to 8.5 μg. during Period III with a sodium intake of 25 mEq. a day, but fell to 4 μg. during the period of maximum sodium restriction in Period IV. The mean of 4 values during a sodium intake of 210 mEq. daily was 3 μg. per twenty-four hours.

In Subject J.F. (Fig. 7) the pattern of adaptation to sodium restriction resembled that observed in J.B., except that the loss of sodium and of weight was of smaller magnitude on the day of reduction from 210 to 70 mEq. of sodium. The overall urinary sodium loss in excess of intake amounted to 163 mEq., with a 1.2-Kg. weight loss. The urinary aldosterone levels increased immediately in response to sodium restriction. The mean 24-hour level of excretion of aldosterone during a daily intake of 210 mEq. of sodium was 4 μg. (4 determinations); it rose to a mean of 17.5 μg. when the sodium intake was reduced to 70 mEq. a day, and further to a mean of 22 μg. when the sodium intake was 25 mEq. a day, but fell to a mean of 18.5 μg. during the last five-day period when the sodium intake was only 8 mEq.

Thus in both subjects exposed to progressive restriction of sodium in-

TABLE 2. ADAPTATION OF 2 NORMAL MALE SUBJECTS TO PROGRESSIVE RESTRICTION OF DIETARY SODIUM

J.B., 28 yrs.
Diet: Sodium (mEq./day) 8
Potassium (mEq./day) 92
Calories/day 2535

Period*	Day	Saliva			Urine (values per 24 hrs.)				
		Body wt. (Kg.)	Na (mEq./L)	K (mEq./L)	Total vol. (ml.)	Creatinine (mg.)	Na (mEq.)	K (mEq.)	Aldosterone (μg.)
I	1				1060	2390	191	84	2
	2	66.9	17	20	1000	1910	227	58	4
II	3	66.9	18	20	1060	1920	224	61	1
	4	66.3	23	18	1760	1830	120	53	5
	5	66.25	18	18	1340	1800	56	67	2.5
	6	66.25	18	19	990	1810	49	75	5.5
	7	66.2	20	21	1040	1550	49	73	7
III	8	66.4	17	20	1340	1780	43	70	14
	9	66.2	9	20	1370	1870	30	88	3
	10	66.1	9	20	1440	1950	29	68	16
	11	66.1	9.6	20	1000	1810	17	64	7
	12	66.1	15	20	1040	1600	18	68	2
IV	13	66.0	15	22	670	1850	9	71	3.5
	14	66.0	12	21	1000	1950	13	72	5
	15	65.9	6.6	19	940	1770	7.3	75	3.5
	16	65.8	13	22	760	1930	5	71	0.5
	17	65.9	12	24	1300	1850	9	60	8
	18	65.6	14.8	20					

(Continued on facing page)

* During Period I, sodium chloride (202 mEq. of sodium) was added to the diet.
During Periods II and III, 62 and 17 mEq. of sodium respectively were, daily, added to the diet.
The diet was taken without salt supplement during Period IV.

take, aldosterone was excreted in the urine in larger quantities during a period when the sodium intake was 25 mEq. a day than when the sodium intake was only 8 mEq. a day, despite an accentuation of the sodium deficit. Moreover, Subjects J.B. and J.F., with comparable control levels of aldosterone excretion, showed similar patterns of excretion of the hormone, although the absolute values were widely different. Both men excreted maximal amounts of aldosterone during Period III, and both excreted comparable amounts in Periods II and IV.

In neither of the subjects studied in this manner was there any signifi-

TABLE 2. (*Continued*)

		J.F., 31 yrs. Diet: Sodium (mEq./day) 8 Potassium (mEq./day) 135 Calories/day 3145							
I	1				970	1920	110	99	6
	2	64.9	8.6	19	1600	1770	175	115	2
II	3	64.9	8.6	19	1730	1800	176	88	11
	4	64.8	12	19	1910	2120	99	115	20
	5	64.8	10	17	1800	1820	73	101	23
	6	64.5	9.4	18	1500	1690	62	104	19
	7	64.7	6.4	21	1220	1630	58	113	15
III	8	64.7	8	18	1400	1910	38	110	16
	9	64.6	7.2	21	1510	1890	27	109	16
	10	64.6	6.5	20	1770	1840	27	105	15
	11	64.3	7.5	19	1420	1950	19	108	46
	12	64.5	9.2	19	1520	1870	23	103	17
IV	13	64.3	9.9	19	1180	1860	20	103	10
	14	64.3	7.1	20	990	1800	24	83	39
	15	64.3	7.4	19	840	1890	11	99	12
	16	64.2	7	19	1340	1990	12.5	95	8
	17	64.2	6.3	20	900	1680	8.4	95	24
	18	63.7	6.3	19					

cant change in the pattern of urinary excretion of potassium during the seventeen days of the diet. On the other hand, both showed a decrease in the salivary sodium/potassium ratio to approximately the same level (0.4). As previously, the changes in this ratio were due solely to variations in the concentration of sodium (Table 2).

When the patterns of adaptation to sudden and to progressive sodium restriction in Subjects J.B. and J.F. are compared, it is apparent that the total weight loss and the total urinary sodium loss are remarkably similar in the 2 types of study. Subject J.B. lost 1.0 and 1.2 Kg. of body weight respectively, with corresponding urinary sodium deficits of 168 and 163 mEq.; J.F. lost 1.4 and 1.2 Kg. of body weight respectively, with corresponding urinary sodium losses of 185 and 163 mEq. in excess of dietary intake. The sodium deficit appears to have been of greater magnitude at the end of the study involving progressive withdrawal of sodium from the diet, if the extrarenal losses of sodium are taken into consideration. During a low-sodium intake, the fecal excretion of sodium is negligible (12); but the "insensible" loss of sodium amounts to about 4 mEq. a day (13), and could easily be two to three times as much in ambulant subjects such as

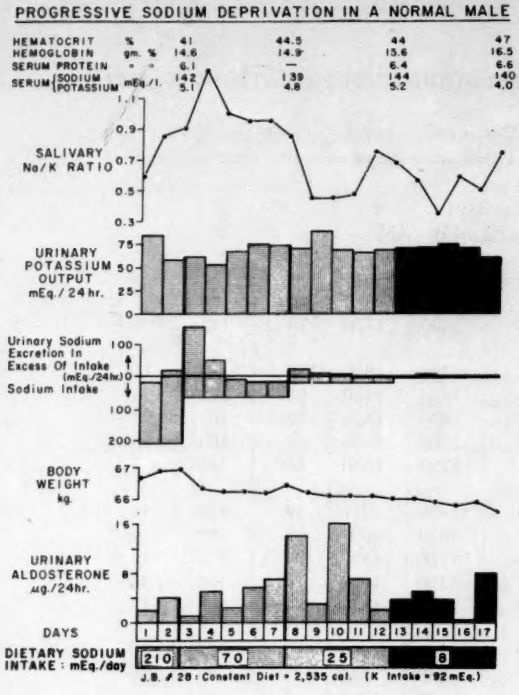
PROGRESSIVE SODIUM DEPRIVATION IN A NORMAL MALE
HEMATOCRIT % 41 44.5 44 47
HEMOGLOBIN gm. % 14.6 14.9 15.6 16.5
SERUM PROTEIN " " 6.1 — 6.4 6.6
SERUM SODIUM mEq./l. 142 139 144 140
SERUM POTASSIUM 5.1 4.8 5.2 4.0
SALIVARY Na/K RATIO
URINARY POTASSIUM OUTPUT mEq./24 hr.
Urinary Sodium Excretion In Excess Of Intake (mEq./24hr.)
Sodium Intake
BODY WEIGHT kg.
URINARY ALDOSTERONE µg./24hr.
DAYS
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
DIETARY SODIUM INTAKE : mEq./day
210 70 25 8
J.B. # 28 : Constant Diet = 2,535 cal. (K Intake = 92 mEq.)

FIG. 6

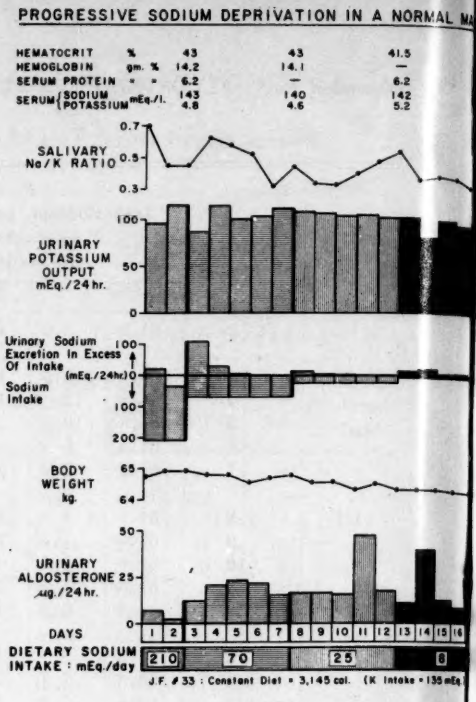
PROGRESSIVE SODIUM DEPRIVATION IN A NORMAL MA
HEMATOCRIT % 43 43 41.5
HEMOGLOBIN gm. % 14.2 14.1 —
SERUM PROTEIN " " 6.2 — 6.2
SERUM SODIUM mEq./l. 143 140 142
SERUM POTASSIUM 4.8 4.6 5.2
SALIVARY Na/K RATIO
URINARY POTASSIUM OUTPUT mEq./24 hr.
Urinary Sodium Excretion In Excess Of Intake (mEq./24hr.)
Sodium Intake
BODY WEIGHT kg.
URINARY ALDOSTERONE µg./24hr.
DAYS
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
DIETARY SODIUM INTAKE : mEq./day
210 70 25 8
J.F. # 33 : Constant Diet = 3,145 cal. (K Intake = 135 mEq.

FIG. 7

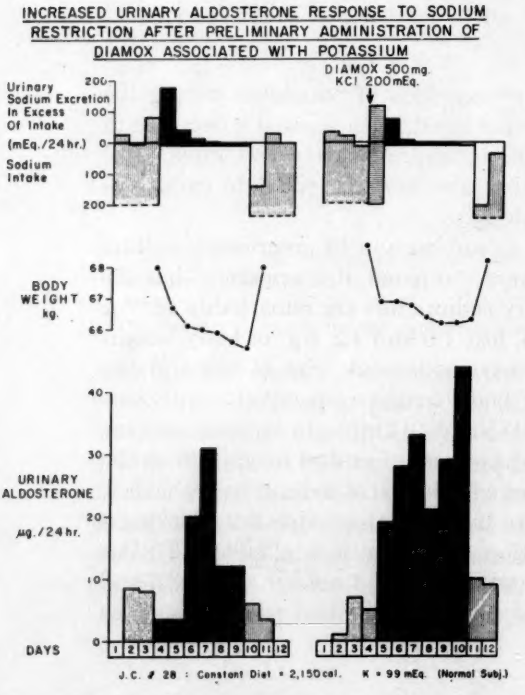
INCREASED URINARY ALDOSTERONE RESPONSE TO SODIUM RESTRICTION AFTER PRELIMINARY ADMINISTRATION OF DIAMOX ASSOCIATED WITH POTASSIUM
DIAMOX 500mg.
KCl 200mEq.
Urinary Sodium Excretion In Excess Of Intake (mEq./24hr.)
Sodium Intake
BODY WEIGHT kg.
URINARY ALDOSTERONE µg./24hr.
DAYS
1 2 3 4 5 6 7 8 9 10 11 12
J.C. # 28 : Constant Diet = 2,150 cal. K = 99 mEq. (Normal Subj.)

FIG. 8. The dotted lines for the periods of high sodium intake mean that the latter is approximate.

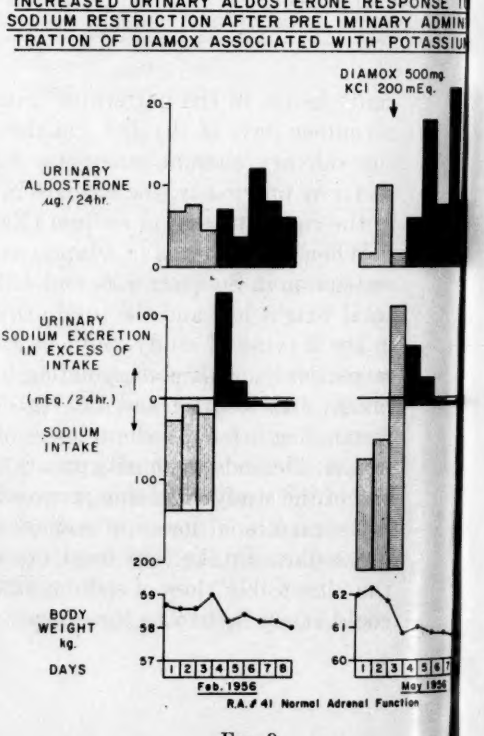
INCREASED URINARY ALDOSTERONE RESPONSE T
SODIUM RESTRICTION AFTER PRELIMINARY ADMIN
TRATION OF DIAMOX ASSOCIATED WITH POTASSIU
DIAMOX 500mg.
KCl 200 mEq.
URINARY ALDOSTERONE µg./24hr.
URINARY SODIUM EXCRETION IN EXCESS OF INTAKE (mEq./24hr.)
SODIUM INTAKE
BODY WEIGHT kg.
DAYS
1 2 3 4 5 6 7 8
Feb. 1956
1 2 3 4 5 6 7
May 1956
R.A. # 41 Normal Adrenal Function

FIG. 9

studied here (14). Despite this, the rise in urinary aldosterone in our study was more marked following abrupt withdrawal of sodium from the diet.

3. *Enhancement of the aldosterone response to dietary sodium withdrawal when combined with the administration of natriuretic agents*

Two subjects with normal adrenal function were maintained on a constant low-sodium diet (9 mEq. per day in R.A., and 10 mEq. per day in J.C.) for five or six days, on 2 separate occasions. On one of these occasions, the period of sodium restriction was immediately preceded by the oral administration of acetazolamide (500 mg. of Diamox taken in 2 doses) combined with 200 mEq. of potassium (as potassium chloride). The potassium was added in order to correct the kaliuretic effect of acetazolamide and to increase its natriuretic action (15).

In both subjects, the total weight loss and the sodium loss were approximately unchanged at the end of the period of sodium restriction, whether or not acetazolamide plus potassium had been given, but the rise in urinary aldosterone was twice as great when this combination of drugs was taken as when the low-sodium diet was administered alone.

Subject J.C. was given the same diet for Period II of both studies (Table 3 and Fig. 8). There was no appreciable difference either in the total amount of weight lost or in the rate of this loss, between the 2 studies. If anything, the total loss of weight and of sodium were more pronounced without prior administration of acetazolamide and potassium. The latter combination followed by the low-sodium diet had induced, at the end of the seven-day study, a cumulative urinary loss exceeding the intake by 179 mEq. for sodium, by 178 mEq. for chloride, and a total weight loss of 2.4 Kg. During the "control" period, the urinary sodium and chloride losses were 230 and 213 mEq., respectively; in keeping with this, the weight loss was slightly more pronounced, being 2.6 Kg.

The urinary output of aldosterone, not elevated on the day of administration of acetazolamide and potassium, started to rise fairly regularly from the following day to the end of the period of sodium restriction. The mean value for the six days of low-sodium diet was 29 μg. per twenty-four hours. When this same diet was given without prior administration of the acetazolamide and potassium combination, aldosterone did not rise before the third day, and began to decrease on the fifth day, averaging only 14 μg. per twenty-four hours for the entire period.

In both instances restoration of sodium to the diet induced an almost quantitative retention of sodium, whereas urinary aldosterone was immediately brought back to normal levels.

In the study performed on Subject R.A. (Table 3, Fig. 9) the low-sodium diet following acetazolamide and potassium administration re-

TABLE 3. ADAPTATION OF 2 MALE SUBJECTS WITH NORMAL ADRENAL FUNCTION TO SODIUM DEPRIVATION INDUCED BY DIETARY MEANS FOLLOWING THE ADMINISTRATION OF ACETAZOLAMIDE AND POTASSIUM

Day	Body wt. (Kg.)	Intake per day				Urinary output (per 24 hrs.)					Aldosterone (μg.)
		Fluid (ml.)	Na (mEq.)	K (mEq.)	Cl (mEq.)	Total vol. (ml.)	Creatinine (mg.)	Na (mEq.)	K (mEq.)	Cl (mEq.)	
Dietary sodium restriction (control study)											
					*1) J.C., 28 yrs., normal male**						
1	68.3	—	200	120	200	1600	1410	213	80	200	—
2		—	200	120	200	1080	1770	182	89	214	8.5
3		—	200	120	200	1180	1620	266	108	282	8.0
4	68.0	1000	10	99	23	1480	1780	188	71	205	3.5
5	66.7	1000	10	99	23	720	1720	48	63	51	3.5
6	66.1	1000	10	99	23	725	1940	22	82	27	20.0
7	66.0	1000	10	99	23	760	1730	12	98	25	31.0
8	65.9	1000	10	99	23	740	1760	11	110	22	12.0
9	65.6	1000	10	99	23	765	1700	9	108	21	12.0
10	65.4	—	236	105	250	1020	1970	80	110	84	7.0
11	68.0	—	236	105	250	1960	1800	250	90	290	3.5
12			236	105	250	1220	1790	218	110	240	—
					2) R.A., 41 yrs., benign spinal cord tumor						
1	58.7	—	138	94	—	2840	1090	111	74	—	6.5
2	58.6	—	138	94	—	1980	1090	128	70	—	7.5
3	58.6	—	138	94	—	1990	1190	115	65	—	4.5
4	59.0	—	9	94	—	2800	815	137	75	—	7.0
5	58.2	—	9	94	—	1980	1145	30	88	—	3.5
6	58.4	—	9	94	—	2050	1065	5.5	80	—	12.0
7	58.2	—	9	94	—	2220	1060	2.0	82	—	8.0
8	58.2	—	9	94	—	2420	1070	3.5	68	—	6.0
9	58.0		9	94	—						
Dietary sodium deprivation after acetazolamide and potassium											
					*1) J.C., 28 yrs., normal male**						
1		—	196	90	200	1000	—	216	99	169	—
2		—	196	90	200	950	—	198	78	208	1.5
3		—	196	90	200	830	1780	168	85	238	8.0
4†	68.7	1500	200	299	413	2320	—	318	302	296	4.5
5	66.9	1500	10	99	23	1100	—	83	174	238	19.0
6	66.9	1500	10	99	23	1320	1110	17	67	72	28.0
7	66.8	1500	10	99	23	1250	1540	5.5	52	36	33.0
8	66.4	1500	10	99	23	1240	1490	6.0	66	28	21.0
9	66.2	1500	10	99	23	1580	1820	5.5	89	35	29.0
10	66.2	1500	10	99	23	1180	1770	4.0	73	24	44.0
11	66.3	—	244	140	250	870	2320	28	96	36	10.0
12	68.2	—	244	140	250	1200	2210	192	116	222	9.5
13			244	140	250	1300	1825	312	103	306	—
					2) R.A., 41 yrs., benign spinal cord tumor						
1	62.2	2300	200	67	208	2560	1035	134	61	180	1.5
2	62.2	2300	200	67	208	2740	1180	179	66	205	10.0
3†	61.8	2300	200	267	408	3620	1220	311	155	260	1.5
4	60.8	2300	9	67	18	2170	1160	75	172	245	6.0
5	61.0	2300	9	67	18	2550	1105	34	61	82	18.0
6	60.8	2300	9	67	18	2500	1080	6.0	55	32	7.5
7	60.8	2300	9	67	18	2340	1100	3.0	47	14	22.0
8	60.8	2300	9	67	18	2430	1035	5.0	56	4.5	8.0
9	—	2300	9	67	18	2130	945	5.5	50	11	13.0
10	60.6										

* In Subject J.C., during Periods I and III the diet was not the same as during Period II; the values given for intake are approximate in these 2 periods.

† To the constant diet were added: 200 mEq. of potassium (as potassium chloride), 190 mEq. of sodium (as sodium chloride), and 500 mg. of acetazolamide.

sulted in a weight loss of 1.2 Kg., and a urinary sodium loss in excess of dietary intake amounting to 180 mEq. Urinary aldosterone, low on the day of diuresis resulting from the administration of acetazolamide and potassium, did not rise significantly before the second day of the low-sodium diet, to reach a maximum on the fourth day. The mean 24-hour excretion of aldosterone was 15 μg. It had been 7.5 μg. during the "control" five-day period of strict sodium deprivation (illustrated on the left-hand side of Figure 9) with a weight loss of 1.0 Kg. and a urinary sodium deficit amounting to 130 mEq.

4. *Estimation of the loss in extracellular fluids consecutive to dietary sodium deprivation*

In normal persons exposed to salt restriction, the sodium excreted in excess of intake is probably derived from the extracellular compartments, with an ensuing isosmotic loss of the latter as long as the sodium deficit does not exceed approximately 400 mEq. (16, 17). Accordingly, in 5 studies, the weight loss resulting from sodium restriction was converted into terms of the percentage of the extracellular volume initially present; the latter was estimated at 23 per cent of the control body weight (bromide space measurement (18)). The values obtained are presented in Table 4, where

TABLE 4. ABSENCE OF CORRELATION BETWEEN THE RISE IN URINARY EXCRETION OF ALDOSTERONE AND THE CHANGES IN THE VOLUME OF EXTRACELLULAR COMPARTMENTS INDUCED BY DIETARY SODIUM DEPRIVATION IN 3 NORMAL MALES

Subjects	Relative loss in extracellular fluid volume (derived from weight loss)*			Relative loss in plasma volume (derived from changes in hematocrit and hemoglobin)†	Urinary excretion of aldosterone (μg. per 24 hrs.)		
	Initial body (Kg.)	Weight loss (Kg.)	ECF loss (%)	%	Control values	Values during minimal sodium intake	
						Mean	Range
J.B.^{S}**	66.9	1.0	6.5	12	3.0	6.5	2–13
J.B.^{P}	66.9	1.3	8.5	18	3.0	4.0	0.5– 8
W.D.^{S}	66.1	1.5	10.0	8	4.5	21.5	6–42
J.F.^{S}	65.3	1.4	9.5	15	4.5	26.0	9–35
J.F.^{P}	64.9	1.2	8.0	—	4.0	18.5	8–39

* Assuming that the extracellular fluid represents 23% of the total body weight (18), and that the weight loss represents only extracellular fluid (16, 17).

† Using the formula

$$\frac{PV_2}{PV_1} = \frac{(1 - Hct_2) \times Hb_1}{(1 - Hct_1) \times Hb_2} \quad (19).$$

** S = Sudden sodium restriction; P = Progressive sodium restriction.

they are compared with those calculated from changes in venous hematocrit and hemoglobin concentration induced by salt restriction. Except in 1 instance, the reason for which is obscure, the observed reduction in plasma volume appears to have been at least as pronounced as the calculated change in extracellular fluid volume.

No close correlation can be drawn between the magnitude of the rise in urinary aldosterone and that of the (estimated) decrease in the size of extracellular compartments, in these 5 studies on sodium deprivation.

DISCUSSION

The observations presented here are noteworthy for several reasons. First, they cannot be completely reconciled with the hypothesis of the control of aldosterone secretion proposed by Bartter (1, 2), according to which there is a direct relationship between the magnitude of the decrease of the extracellular volume and that of the rise in urinary aldosterone excretion. Secondly, these observations show that a considerable reduction in the urinary excretion of sodium can occur when a normal subject is given a sodium-restricted diet despite absence of any definite increase of aldosterone excretion. Thirdly, there are wide individual variations in the magnitude of the aldosterone response to sodium restriction. Fourthly, and arising from the foregoing, these observations do not confirm the quantitative inverse relationship said to exist between the sodium excreted in the urine and the urinary aldosterone levels (20, 21).

Because of the limitations of the method for measurement of aldosterone in urine (10), patterns of aldosterone excretion and mean levels are considered in this study rather than placing undue emphasis on the results of single determinations. The reasons for assuming that changes in the amount of aldosterone in the urine reflect quantitatively changes in the rate of production of the hormone by the adrenal cortex have already been discussed (5).

When the normal subjects were submitted to dietary restriction of sodium, the major part of the weight loss occurred on the first day of sodium restriction, whereas the maximum excretion of aldosterone was achieved on this day only in 1 case out of 11; in the majority of cases maximal excretion was achieved on the third or fourth day, and in 2 cases, only on the last day. Seemingly, the more sudden the weight loss, the greater the rise in aldosterone excretion. In 2 subjects, the mean level of aldosterone excretion when the dietary intake of sodium was dropped from 210 to 8 mEq. by means of progressive salt withdrawal was not as great as when this reduction was made abruptly (compare Figs. 3 and 6, and 5 and 7). Conversely, in 2 other subjects, the administration of diuretics on the day before beginning the low-sodium diet induced a brisker and greater al-

dosterone response than that observed during the control period when no diuretic was given (Figs. 8 and 9). These differences in the magnitude of the aldosterone response to sodium deprivation were obviously not a direct quantitative reflection of variations of either the loss of sodium or the loss of weight.

In only 2 of the 11 studies did the level of aldosterone excretion increase progressively up to the last day of sodium restriction. Despite this, in all cases the daily sodium loss (renal plus extrarenal) always exceeded the intake, resulting in an accentuation of the deficit of sodium, and presumably of the decrease of extracellular volume. A striking illustration of this is given by the study on the progressive withdrawal of sodium, in which the levels of excretion of aldosterone were less during the period of maximal sodium restriction (daily intake, 8 mEq.) than during the preceding period when the sodium intake was 25 mEq. per day. The assumption must therefore be made that the controlling mechanism for aldosterone secretion, if it be a volume receptor, lies in some special area of the extracellular compartment and that a redistribution of body fluids leads to preservation of the volume of this compartment from the depleting effect of sodium loss.

Expansion of the extracellular compartment follows the administration of many corticosteroids in acute experiments in animals (22, 23) and in man (24). If aldosterone, in the quantities circulating during the studies on sodium restriction, has this property, the decrease of the extracellular compartment may actually be smaller than is suggested by the observed losses of weight and of sodium. Under the influence of the hypersecretion of aldosterone secondary to sodium deprivation, an internal redistribution of fluids (resulting in a re-expansion of all, or some, of the extracellular compartments) might be invoked to explain why the rate of secretion of aldosterone, after reaching a maximum during sodium deprivation, becomes stabilized at a lower level at the time of maximal sodium retention. It has been suggested (1) that the blood volume is more important than the extracellular space, as a whole, in the regulation of aldosterone secretion. However, calculations of changes in plasma volume made in 5 instances (Table 4) show that in 3 cases the decrease in plasma volume was proportionally greater, if anything, than the estimated loss of extracellular volume. Conversely, in 1 subject (J.F.) in whom there was no apparent change in plasma volume, aldosterone excretion was definitely elevated.

The quantitative inverse relationship reported as existing between the level of excretion of aldosterone and the amount of sodium excreted in the urine (20, 21) is not supported by these observations under conditions of changing sodium intake. The urinary sodium output continued to decrease as the result of dietary deprivation of sodium, regardless of the pattern of

aldosterone excretion. The use of the logarithm of the urinary potassium/sodium ratio proposed by Luetscher (25) would not be more helpful, since the urinary excretion of potassium likewise did not exhibit any relationship with this pattern. Similarly, when sodium was restored to the diet there was an almost quantitative retention of sodium on the first day, although aldosterone excretion fell to normal, or near normal, levels.

Aldosterone probably stimulates the reabsorption of sodium in the renal tubule (26); this is also a property of certain other corticosteroids (27). If there be a quantitative relationship between the amounts of aldosterone found in the urine and the circulating levels, as well as between the concentration of biologically active aldosterone in the body and the extent of its effects on the reabsorption of sodium, it seems surprising that the sodium retention during sodium restriction becomes more intense at the time that urinary aldosterone values are decreasing. A direct correlation has been found repeatedly between the intensity of the sodium retention by the kidneys of adrenalectomized animals and the dose of aldosterone injected, and is indeed the basis of biologic methods for the measurement of aldosterone (28–30). This correlation exists in acute situations in which the load of sodium delivered to the tubule could be assumed to be constant throughout the experiment; but it is possible that, after a few days of sodium deprivation, other factors come into play, resulting in a diminution of the amount of sodium left available for the process of active tubular reabsorption depending on aldosterone. The filtered load of sodium might decrease as a result of a fall in the concentration of serum sodium (which was observed in all 5 cases in which this determination was made) combined or not with a diminished glomerular filtration rate. A significant reduction in glomerular filtration in normal persons exposed acutely to profound restriction in sodium intake has never been established (3, 31) unless combined with large extrarenal losses of this electrolyte (16). But Black *et al.* (32) in man and Frieden *et al.* (33) in the dog have demonstrated that there is in fact an increased sodium reabsorption by the kidney tubules during low sodium intake.

When such observations are correlated with those reported here concerning the excretion of aldosterone, the possibility should be considered that the absence of a close quantitative relationship between the excretions of aldosterone and sodium under conditions of sodium deprivation may signify the existence of "permissive" action of the hormone on sodium reabsorption by the renal tubule. However, deoxycorticosterone in a dosage of 10 mg. daily would not seem to have such a property (5).

There are more suggestions that factors other than aldosterone concentration are normally concerned with the renal retention of sodium: (a) The observation was made during this study that resumption of a normal

salt intake resulted initially in an almost quantitative retention of sodium, presumably with a rapid restoration of the pre-restriction extracellular volume, as indicated by the weight gain at a time when aldosterone excretion was generally not higher than during the control days. (b) Leaf *et al.* (34) were able to induce an excessive excretion of sodium by overhydrating subjects, one of whom was suffering from Addison's disease and being maintained with constant doses of cortisone; Weston *et al.* (35) in man, and Cheek and West (36) in experimental animals, failed to inhibit this sodium diuresis by administration of deoxycorticosterone.

There were wide individual variations in the extent of the rise in aldosterone excretion in response to sodium restriction. There did not seem to be any close relationship between the overall or the maximum aldosterone response to dietary sodium deprivation and the extent of the (estimated) loss of extracellular volume induced by the latter, when different subjects were compared (Table 4).

No rise in aldosterone excretion was observed on the day of administration of acetazolamide; this confirms previous data from this laboratory (10) but is not in agreement with the findings of Falbriard *et al.* (37). The reason for the discrepancy is not clear, especially since the drug was administered in conjunction with a fairly large load of potassium, believed to stimulate by itself the urinary excretion of aldosterone (38, 39). In agreement with Falbriard *et al.* (37), however, a definite rise in urinary aldosterone was observed as a result of a sudden dietary restriction of sodium from a high to a moderate level of intake, and not only when minimal quantities of sodium were administered.

It is obvious, from the data presented, that the salivary sodium/potassium ratio does not reflect directly the level of circulating aldosterone as judged by the amount excreted in the urine. The concentration of sodium in the saliva follows the same trend as the urinary excretion of this electrolyte but in a less pronounced and predictable fashion.

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DIVERGENCE IN MAGNESIUM, SODIUM AND POTASSIUM EXCRETION DURING STIMULATION OF ENDOGENOUS ALDOSTERONE PRODUCTION*

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ABSTRACT

Because of reports of hypomagnesemia and magnesium deficiency in subjects with primary aldosteronism, the effect upon magnesium excretion of procedures known to alter aldosterone secretion has been investigated. During periods of of sodium restriction and repletion, urinary exeretion of magnesium, sodium and potassium was studied in 4 normal subjects and in 1 patient with cirrhosis of the liver. In 2 of the normal subjects similar studies were conducted during potassium restriction and repletion.

Increased excretion of aldosterone (sodium-retaining factor) was observed during sodium restriction in the 2 subjects so studied, and in 1 of 2 subjects studied during potassium loading. Despite marked changes in sodium and potassium excretion, urinary magnesium was not consistently altered.

It is concluded that under the conditions of this study, urinary magnesium is not altered by stimulation of endogenous aldosterone production. Evidence is cited which suggests that magnesium deficiency in patients with primary aldosteronism may be due to the renal dysfunction associated with potassium deficiency.

FOLLOWING the original description of primary aldosteronism by Conn (1), a number of such cases have been reported. In addition to the usual findings of hypokaliemia, alkalosis, tetany and mild hypertension, Mader and Iseri (2) noted the occurrence of low serum magnesium levels in a case of this syndrome. In this patient, increased loss of magnesium was observed in both urine and stool and it was concluded that a defect in renal tubular reabsorption of magnesium was present. Although the effects of aldosterone upon sodium and potassium metabolism have been well described, the observations upon magnesium metabolism have been confirmed in only 1 other case of primary aldosteronism (3). Low serum

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magnesium concentrations have been observed, however, in patients with hepatic cirrhosis (4), a condition in which, when associated with ascites, increased urinary excretion of aldosterone has been found (5). These observations have stimulated our interest in studying the effects of aldosterone on the excretion of magnesium.

Because no aldosterone was available for administration, it was necessary to cause alterations in endogenous aldosterone secretion in order to study its effects upon magnesium excretion. Increased urinary aldosterone excretion has been demonstrated in normal subjects following sodium deprivation (6), following a decrease in extracellular fluid volume (7, 8), and during potassium loading (9). In an attempt to determine the effect of increased aldosterone secretion upon renal handling of magnesium, studies were performed in subjects who were maintained on dietary regimens designed to cause an increase in endogenous aldosterone production.

MATERIALS AND METHODS

Four normal subjects and 1 patient with hepatic cirrhosis were studied during periods of varying sodium or potassium intake. The diets used for all studies are shown in Table 1.

Two normal subjects (M. H., female and Bl., male), received low-sodium and low-potassium diets. During the periods of low or high sodium intakes, in order to provide variety, 2 diets low in sodium and nearly constant in magnesium were provided; each was used on alternate days. During the periods of low or high potassium intakes, the same diet was given daily. Increases in sodium or potassium intakes were accomplished by giving sodium chloride or potassium salts[1] in addition to the diet.[2]

Two other normal subjects (E. R., female and B. L., male) and a patient (E. A., male) with cirrhosis who was undergoing spontaneous diuresis at the time of study, all received identical diets, with the same menu given daily. This diet contained 8.0 mEq. of sodium, and sodium chloride was given to raise the total intake of sodium to 94.0 mEq. per day during the high-sodium intake periods. The first three days were used to allow adaptation to the diet.

The serum of each normal subject was analyzed for sodium and potassium at the beginning of each new study period. In addition, the serum magnesium level was determined at the same intervals in Subject E. R. Consecutive 24-hour urine collections were analyzed for sodium, potassium and magnesium.

The dietary intake of protein, calories and magnesium was calculated from standard tables (10). The sodium and potassium content was determined by wet ashing and flame photometry, using internal lithium standards. Serum and urine were analyzed for magnesium by a modification of the titan yellow method (11). Aldosterone was determined by bio-assay (12). Since this technique measures only the presence of sodium-retaining factors, the values obtained are referred to as such, expressed as desoxycorticosterone acetate equivalents. These values undoubtedly include aldosterone as the major sodium-retaining factor.

[1] Potassium chloride and potassium Triplex Solution (potassium acetate, bicarbonate and citrate) were kindly supplied by Eli Lilly and Company, Indianapolis, Indiana.

[2] Lonalac® for use in the low-sodium diets was kindly supplied by Mead Johnson and Company, Evansville 21, Indiana.

TABLE 1. DIETS

Diet	Calories	Protein (Gm.)	Sodium (mEq./day)	Potassium (mEq./day)	Magnesium (mEq./day)
Diet for Subjects M.H. and Bl.					
Low-sodium #1*	2020	78.0	8.0	75.0	19.3
Low-sodium #2	2018	78.0	8.0	75.0	19.8
Low-potassium	1903	67.0	80.0	11.0	constant, unknown†
Diet for Subjects E.R., B.L., and Patient E.A.					
Low-sodium	2207‡	80.4	8.0	92.6	23.1

* Low-sodium diets 1 and 2 were given on alternate days.

† Magnesium values are unavailable for the foods used in this diet.

‡ In the diet of E.R.. there was an addition of 343 calories from sugar.

RESULTS

The urinary excretion of sodium in all 5 subjects followed the expected pattern of decrease during the reduction of sodium in the diet (Figs. 1–5). Likewise, urinary potassium fell during the period of low potassium intake and rose with potassium supplementation in the 2 subjects so studied (Figs. 1 and 2). Despite the changes in the excretion of these ions, magnesium excretion was not markedly altered. Urinary magnesium excretion was low on the third day of sodium restriction in Subject M.H. but no similar change was seen in the other subjects. A slight decrease in urinary magnesium occurred during the period of potassium repletion in Subjects M.H. and Bl., but this may have been a reflection of a lower magnesium intake while receiving the low-potassium diet.

In the patient with cirrhosis, E.A. (Fig. 5), who was undergoing spontaneous diuresis at the time of study, urinary sodium was in excess of intake during all 3 periods. Although the urinary sodium fell during the low-sodium intake period, it did not reach as low a value as noted in the other subjects studied. Despite this failure of the sodium excretion to fall as sharply as in the normal subjects, the cirrhotic patient's magnesium excretion was not different from that in the normal group.

Urinary sodium-retaining material rose in response to a low sodium intake and decreased in association with sodium repletion in both M.H. and Bl. No change was observed during the low-potassium period, but in M.H. the excretion of sodium-retaining factor rose during the period of potassium repletion (Fig. 1).

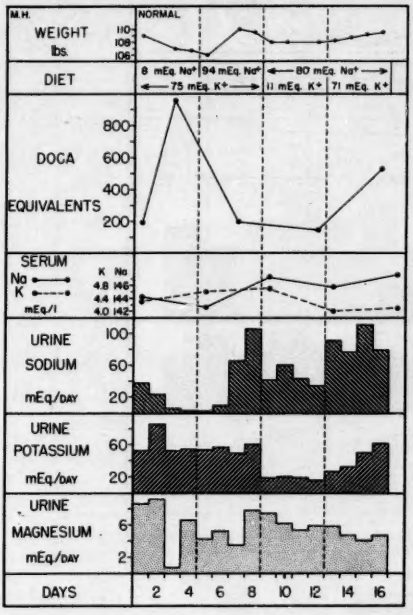

FIG. 1. M.H. (female). Electrolyte excretion during successive periods of 1) low sodium intake, 2) sodium supplement, 3) low potassium intake, and 4) potassium supplement. DOCA equivalents indicate urinary excretion of aldosterone or sodium-retaining factor.

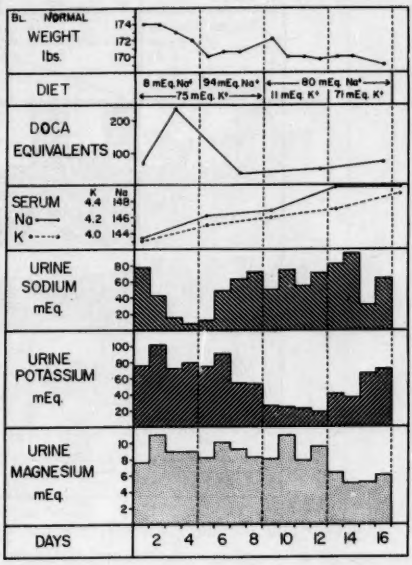

FIG. 2. Bl. (male). Same experimental sequence as for M.H.—see Figure 1.

Serum sodium and potassium concentrations were not altered significantly, although the levels of both ions rose in Subject Bl. In all normal subjects, there were slight changes in weight which were correlated with the changes in sodium intake. In Patient E.A., the occurrence of spontaneous diuresis led to a progressive decrease in weight as the loss of ascitic fluid continued.

Serum magnesium concentration in Subject E.R. showed slight but insignificant changes during the study. The values for days 1, 4, 8 and 12 were 2.3, 2.4, 2.7 and 2.4 mEq. per liter, respectively.

DISCUSSION

The data presented show that despite marked changes in sodium and potassium excretion with associated changes in aldosterone excretion, there was little or no alteration in magnesium excretion. Although sodium-

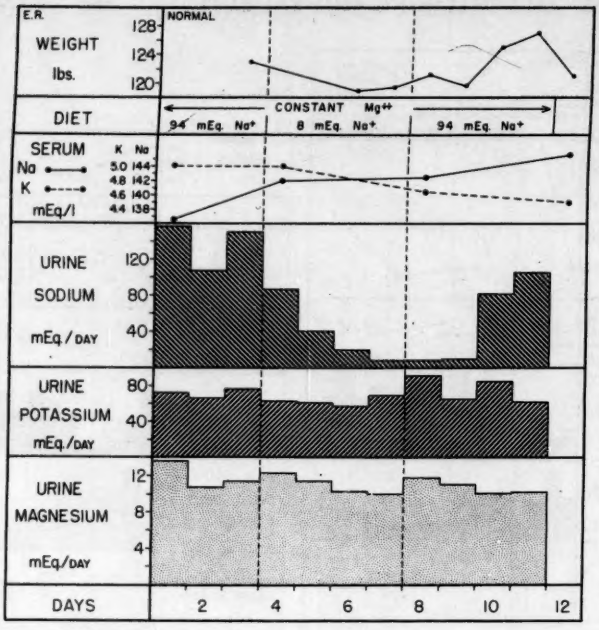

FIG. 3. E. R. (female). Electrolyte excretion during successive periods of 1) normal sodium intake, 2) low sodium intake, and 3) normal sodium intake.

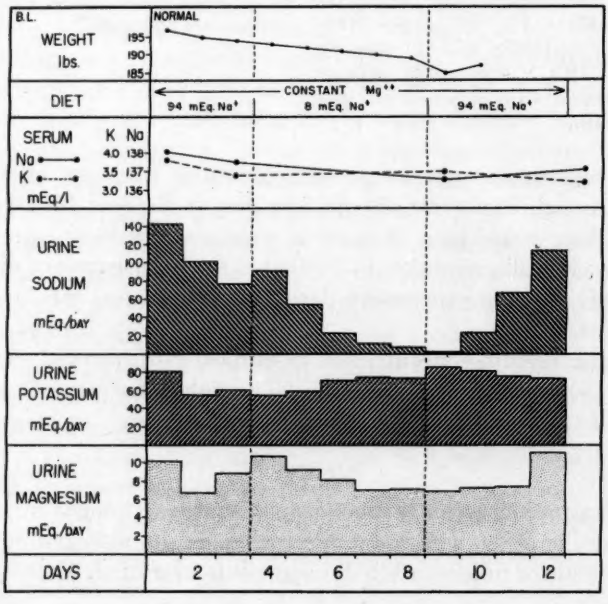

FIG. 4. B. L. (male). Same experimental sequence as for E. R. (see Fig. 3), except for a five-day low-sodium period.

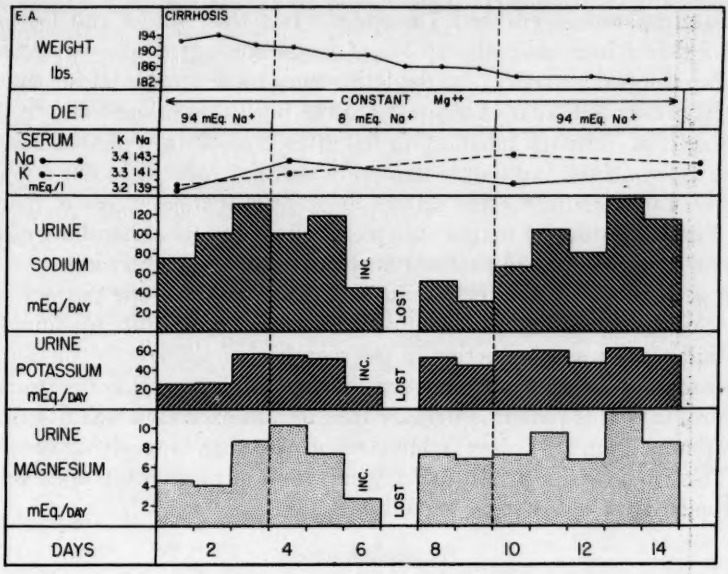

FIG. 5. E. A. (male, cirrhosis). Same experimental sequence as for E. R. and B. L. (Figs. 3 and 4), except for a six-day low-sodium period and a subsequent five-day period of normal sodium intake. Note that urinary sodium excretion is in excess of intake in all periods.

retaining factor was not measured in 3 of the subjects studied, it is probable that it rose in response to the low intake of sodium, since this has been consistently observed by others (6, 13, 14).

These findings are not in accord with the observations of Mader and Iseri (2) and of Milne, Muehrcke and Aird (3), who found magnesium deficiency in patients with primary aldosteronism. There were significant differences between their study and ours, however, both in the conditions and in the physical status of the subjects. These differences were chiefly related to the degree and chronicity of the aldosterone secretion and the possible effect of long-standing potassium depletion. The degree of stimulation of aldosterone secretion, although comparable to levels observed in some patients with aldosteronism (15, 16), may not have been as great in our subjects as in theirs. This, and the relatively short duration of stimulation in our subjects, may have accounted for the lack of effect.

Of possibly greater importance may be the role of potassium in explaining the occurrence of magnesium loss in the reported patients. Potassium depletion has been demonstrated to produce changes in renal function and histologic structure (17, 18) which may persist for a significant period after

potassium repletion is effected. Despite the fact that Mader and Iseri observed a defect in renal reabsorption of magnesium even after potassium repletion, the residual effects of depletion may have accounted for the abnormality observed. It is of interest that in both this patient and in that of Milne, *et al.*, urinary magnesium fell after potassium repletion had occurred. By contrast, potassium depletion was not present in our subjects and this may account for the failure to observe increased loss of magnesium. Further studies of magnesium metabolism and renal handling of this ion in potassium-depleted patients may prove to be enlightening.

The occurrence of sodium loss in excess of intake in the patient with hepatic cirrhosis, during both sodium restriction and sodium loading, suggests that there was a reduction in the secretion of aldosterone or that sodium-losing factors (19) were present during this period. It is noteworthy, therefore, that this patient's urinary magnesium excretion was not markedly different from that observed in the normal subjects receiving the same diet. This would suggest that urinary excretion of magnesium does not reflect the state of aldosterone secretion.

Acknowledgments

The authors are grateful to Miss Marthana Hjortland, M.S. for the preparation and administration of the diets used in these studies and to Misses Mary Kearney and Joanne Hannye for technical assistance.

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THE URINARY EXCRETION OF INTERSTITIAL-CELL AND FOLLICLE-STIMULATING HORMONE ACTIVITY DURING THE NORMAL MENSTRUAL CYCLE*

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ABSTRACT

Changes in the day-to-day excretion of ICSH have been determined qualitatively throughout 17 menstrual cycles in 13 normal adult women by means of a technique which depends upon the repair of ventral prostatic atrophy in in the hypophysectomized immature male rat. Changes in the excretion of FSH were estimated from the concomitant changes in testicular weight. Composite curves were derived by summating the average deviations from the mean of each individual cycle according to: (a) the days of the cycle, (b) the days preceding or following the day of minimum basal body temperature, and (c) the days prior to the next menstrual period. The quantitative relations between the day-to-day variations in the weights of the ventral prostate and testis were examined by computing regression equations, expressing the composite change in prostatic weight per unit change in testicular weight.

It is concluded that the normal menstrual cycle is characterized by: 1) A mid-cycle peak period of FSH and ICSH excretion, during the early phase of which there is a change in the ratio of ICSH to FSH and a transient preponderance of ICSH. The onset of the mid-cycle peak is closely related in time to the shift in basal body temperature. 2) A late luteal trough followed by a rising trend in the excretion of both hormones, beginning toward the end of the luteal phase and continuing into menstruation.

THE purpose of this communication is to describe the pattern of urinary interstitial-cell stimulating hormone (ICSH) excretion during 17 menstrual cycles in 13 normal adult women. The data were obtained by means of a technique for identifying pituitary ICSH which depends upon

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the repair of ventral prostatic atrophy in the hypophysectomized immature male rat (1). The weights of the rat testes were also recorded as a crude index[1] of follicle-stimulating hormone (FSH) excretion. For reasons stated in an earlier paper (2), we have elected to interpret our data in terms of the dualistic nomenclature which implies that ICSH and FSH exist as independent entities.

METHODS

Healthy female volunteers whose ages ranged from 21 to 40 years served as subjects. All had a normal menstrual history and normal findings on general physical and pelvic examination. The urine was collected in serial 24-hour specimens throughout the cycle and the basal body temperature was recorded daily. Creatinine determinations were performed as a check against gross errors of collection.

Despite its limitations, a single-dose assay of individual specimens was preferred over a multiple-dose assay of pooled specimens, since a major purpose of the study was to relate large differences in the day-to-day excretion of ICSH to other physiologic parameters of the menstrual cycle. According to a method previously described (2), an 18-hour aliquot of each day's specimen was concentrated by means of kaolin adsorption and the equivalent of six hours of urine was injected into each of 3 rats; *i.e.*, the extract was tested for the presence of 4 rat units per twenty-four hours.

The remaining six-hour aliquot of the 24-hour specimen was reserved for determination of the pregnanediol content by a modification of the method of Astwood and Jones (5). In some instances, spot determinations only were performed to establish the presence of physiologically-significant amounts of pregnanediol; in others, daily determinations were made throughout the luteal phase of the cycle. It is recognized that none of the commonly-employed indices of ovulation is infallible. However, inasmuch as pregnanediol appeared in the urine and a mid-cycle shift in basal body temperature occurred in all instances, it seems reasonable to presume that these cycles were ovulatory in character.

RESULTS

A. *Individual patterns*

In Table 1 are summarized the ages of the subjects, their marital status, the lengths of the cycles studied and the percentage of test days on which FSH and ICSH were excreted in amounts sufficient to elicit statistically significant increments in testis and prostate weight. Geometric mean testicular weights of 175.0 mg. or more and prostatic weights of 9.6 mg. or more, based on measurements in 3 rats, were regarded as differing significantly from the control weights ($P = 0.025$)—the geometric mean testicular

[1] According to the studies of Shedlovsky *et al.* (3), pituitary ICSH is able to increase the weight of the testis by approximately 60 per cent, in consequence of ICSH-induced hypertrophy of the Leydig cells and enlargement of the tubules in response to androgen secreted by the interstitial tissue. Additional data (1,4) indicate that the testis does not respond to combinations of pituitary FSH and ICSH with an augmentation reaction. Although many assumptions are clearly required, the qualified use of testis weight as an indicator of FSH effect thus appears justifiable.

TABLE 1. THE AGE AND MARITAL STATUS OF THE SUBJECTS, THE LENGTHS OF THE CYCLES STUDIED, AND THE PERCENTAGE OF DAYS TESTED ON WHICH THE TESTIS AND VENTRAL PROSTATE WEIGHTS OF THE TEST ANIMALS SIGNIFICANTLY EXCEEDED THOSE OF THE CONTROL ANIMALS

Subject	Age	Marital status	Parity	Length of cycle	Percentage of days on which statistically significant* increments in ventral prostatic weight occurred	Percentage of days on which statistically significant** increments in testicular weight occurred
A.C.	21	S	—	32	97	94
M.C.	22	S	—	29	50	90
G.G.	25	S	—	27	85	93
P.I.	33	M	5	35	96	82
J. (a)	38	S	—	26	62	100
(b)	40	S	—	24	52	76
(c)	40	S	—	27	58	88
M.M.	23	S	—	28	88	92
E.P.	26	S	—	27	93	89
M.P.	24	M	0	28	96	100
J.R.	25	S	—	30	92	100
B.S.	21	S	—	29	71	89
Ba.S. (a)	27	S	—	28	88	96
(b)	27	S	—	27	81	81
C.S.	26	S	—	32	84	88
M.W. (a)	26	S	—	33	100	100
(b)	26	S	—	32	100	97
Mean	28			29	82	91
Mean (exclusive of J.)	25			30	87	92

* $\text{Mean}_{(g)}$ prostate weight ≥ 9.6 mg.
** $\text{Mean}_{(g)}$ testis weight ≥ 175.0 mg.

weights for 336 control animals being 148.6 mg. (in logarithms, 2.172 $\pm$0.062) and the geometric mean prostatic weights for 339 control animals being 7.2 mg. (in logarithms, 0.86 $\pm$0.11).

For the majority of subjects significant increases in rat testis weight were observed on a slightly higher proportion of days than were significant increases in prostate weight. During 4 cycles (in Subjects M.C. and J.) the difference in favor of FSH activity was much more pronounced. It may be significant that 3 of these 4 cycles occurred in one subject (J.), the oldest volunteer in the group. Although she was menstruating regularly and, by the available criteria, was ovulating, it is possible that she had begun to manifest the increase in FSH relative to ICSH thought by many to be characteristic of the menopause. In Table 1, if the data for Subject J. are excluded, any difference between the percentages of days on which FSH and ICSH activity were detectable tends to disappear.[2]

If those days on which the rat testicular and prostatic responses exceeded the geometric mean of a given subject's cycle by more than twice the standard error[3] be arbitrarily designated as days of maximal excretion, another criterion of significance ($P = 0.025$) taken from a different base is acquired. In Table 2 are indicated the days of the cycle (expressed as days prior to the next menstrual period) on which the excretion of FSH and ICSH activity reached such maximal levels.

It will be seen that the total number of days on which such levels were attained in any given cycle varied from three to eight for FSH and from three to seven for ICSH activity. The most striking feature of the individual patterns was a period of peak FSH and ICSH excretion during midcycle. The number of consecutive days of maximal excretion at this time varied from one to four. On the day immediately prior to menstruation, maximal rates of FSH excretion were observed during 8 cycles and of ICSH excretion during 6 cycles. During menstruation itself at least one day of maximal FSH excretion was observed during 9 cycles and of ICSH excretion during 5 cycles. These latter observations are in agreement with those of Werner (6), who assayed urinary gonadotropin by the mouse uterine weight method. They seem especially significant because they were obtained despite the potentially toxic effects of menstrual blood which, notwithstanding all precautions, occasionally contaminated the specimens. The data for 1 of the 2 sets of consecutive cycles (Subject Ba.S.) which were secured in this study suggest that the menstrual elevation may repre-

[2] Since the exclusion of this subject was found to exert an imperceptible effect upon all subsequent values, her data were retained in the study.

[3] This standard error was calculated from the standard deviation of the organ weights of the control animals. The number of test animals for each individual per day was assumed to be 3.

TABLE 2. DAYS PRIOR TO THE NEXT MENSTRUAL PERIOD ON WHICH INCREMENTS IN TESTIS OR VENTRAL PROSTATE WEIGHT EXCEEDED TWICE THE STANDARD ERROR*

Subject	Length of cycle		Day																															Total	
			35	34	33	32	31	30	29	28	27	26	25	24	23	22	21	20	19	18	17	16	15	14	13	12	11	10	9	8	7	6	5	4	3	2	1	Testis	V.P.
A.C.	32	Testes									x							x		[ ]		x		x					x								x	5	
		V.P.									x											x						x									x		5
M.C.	29	Testes												x					x					x	x												x	5	
		V.P.									x													x	x												x		4
G.G.	27	Testes												x		x		x				x																4	
		V.P.																				x		x	x		x			x									5
P.I.	35	Testes				x		x								x	x	x	[ ]		x	x	x															8	
		V.P.				x															x	x	x	x	x											x				7
J.(a)	26	Testes													x					[ ]		x	x				x	x								x		6	
		V.P.																				x	x	x			x												4
(b)	24	Testes																		[ ]			x			x	x	x				x					x	5	
		V.P.																					x									x					x		3
(c)	27	Testes									x									[x]		x	x				x		x								x	7	
		V.P.																		[ ]	x	x	x																3
M.M.	28	Testes																x		[ ]		x	x	x	x			x										6	
		V.P.															x						x	x	x			x											5
E.P.	27	Testes									x										[ ]	x	x	x												x	x	6	
		V.P.																			x	x	x	x															4
M.P.	28	Testes											x	x		x				[ ]																x		4	
		V.P.											x		x								x														x		4
J.R.	30	Testes								x		x				x																					3	
		V.P.										x							x				[ ]		x	x	x												5
B.S.	29	Testes												x	x							x	[x]														4	
		V.P.																				x	[ ]		x													3
Ba.S.(a)	26	Testes																				[ ]		x	x	x	x										x	5	
		V.P.																					x	x	x	x										x	x		6
(b)	27	Testes											x									[ ]			x	x			x	x		x			x	x	8	
		V.P.										x						x					x		x	x			x						x				7
C.S.	32	Testes										x		x								[ ]		x				x		x							7	
		V.P.											x										x	x	x					x							x		6
M.W.(a)	33	Testes																					[x]														1	
		V.P.																				x	[ ]	x			x	x											5
(b)	32	Testes				x																[x]						x								x	4	
		V.P.																				x			x			x	x									5
Total		Testes	0	0	0	2	0	1	0	1	3	2	2	5	2	4	1	4	1	1	1	8	9	6	4	3	4	5	2	2	1	0	1	0	0	1	8	88	
		V.P.	0	0	0	1	0	0	0	0	2	2	2	0	1	0	1	1	1	0	3	9	13	9	10	3	5	3	3	2	0	1	0	0	2	1	6		81

* ¦ End of menses.

[ ] Day of minimum basal body temperature.

sent the terminus of a rising trend which began during the late luteal phase of the preceding cycle. A large number of consecutive cycles would be required to validate such an interpretation.

The patterns of FSH and ICSH excretion appear to remain fairly constant from cycle to cycle in a given individual. At least this proved to be the case in 2 subjects (Ba.S. and M.W.) during 2 consecutive cycles, and in another subject (J.) in whom 3 cycles were studied at intervals over a period of three years. In Figure 1 the excretion patterns of Subject J. are superimposed, together with the corresponding basal body temperature curves.

In their general configuration the individual curves are strikingly similar to those depicted by investigators assaying urinary gonadotropin excre-

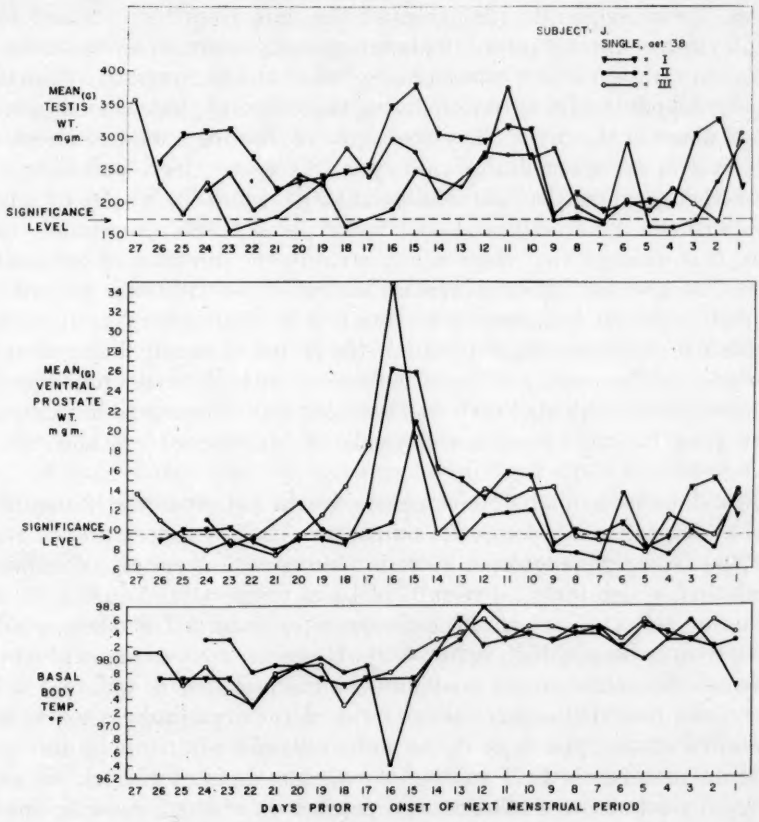

FIG. 1. The changes in testis and ventral prostate weight induced by extracts of urine collected from 1 subject during 3 cycles at intervals over a period of three years.

tion by techniques employing rat ovarian histology (7), rat uterine and ovarian weight (8–18), and mouse uterine weight (6, 19–21). Still earlier investigators, who employed as indices mouse ovarian histology (22) and rat vaginal cytology (23, 24), had obtained positive responses in mid-cycle (24) and just prior to menstruation (23). Venning and Browne (24) were successful in demonstrating the presence of gonadotropin during mid-cycle in only 4 of 10 subjects studied. However, they made the significant observation that gonadotropin became detectable one day prior to mittelschmerz and ovulation bleeding in 2 subjects, and two to three days prior to the appearance of pregnanediol in the urine.

In a study employing rat ovarian weight as the biologic criterion, Albert (25) did not observe a sharp peak in gonadotropin excretion during mid-cycle, although negative values were more infrequent at this than at other times. He subsequently (26) grouped the data from his own and other studies into equal thirds for calculation of mean excretion levels during the follicular, mid-cycle and luteal phases. All of the data agree in showing a slightly higher level of excretion during the follicular phase than during the luteal phase of the cycle. They also agree in showing a modest increase in the level of excretion during mid-cycle. Since, as Albert points out, his method of grouping the data tends to reduce the mid-cycle value by admixture with the lower follicular and luteal phase levels immediately adjacent, it is unlikely that there is any error in the direction of overestimation. The greatest relative increase during mid-cycle (two- to fourfold) quoted in Albert's analysis was obtained in the authors' study (2), in which a preliminary assay to delimit the period of maximal excretion was run prior to the pooling of the urine concentrates. It seems probable that more sensitive methods which enable multiple-dose assays to be performed with a minimum of pooling will yield still higher relative values during mid-cycle.

Few data are available for comparison with respect to the timing of the mid-cycle peak in relation to the shift in basal body temperature. D'Amour (11) measured gonadotropin excretion along with a variety of indices of ovulation, which included records of basal temperatures during 15 menstrual cycles of 4 subjects. The incidence of abnormal cycles appears to have been unusually high in his study. However, a close temporal relation between the gonadotropin peak and the thermal shift is apparent in 7 of the cycles. Lloyd *et al.* (27) noted a rise in the gonadotropin excretion of 1 normal subject just prior to the shift in basal body temperature.

If the increases in FSH and ICSH excretion during mid-cycle are physiologically related to ovulation, the parallelism of the ascending limbs of the curves with that of the mid-cycle rise in basal body temperature could be taken to imply that ovulation generally occurs somewhat later than the

thermal nadir. Such an interpretation would be in essential accord with the observations of Buxton and Engle (28) and of Greulich *et al.* (29). Buxton and Engle made a careful examination of the ovaries during 18 laparotomies timed to coincide with a substantial rise in temperature, whether occurring suddenly from a previous minimum or gradually from a rising baseline. In 6 instances, ovulation had not occurred; in 12 there were corpora lutea, the estimated age of which varied from twelve to seventy-two hours. In 15 women whom Greulich operated upon when the temperature was either rising toward the higher levels characteristic of the luteal phase or had already attained those levels, ovulation was found to have taken place in all instances; in 6 operated upon before the basal temperature had risen, ovulation had not occurred in any case.

B. *Composite pattern*

Attempts to summate the individual cycles are hampered by 2 difficulties: 1) the variation in the level of hormone activity excreted, even during comparable phases of the cycle, and 2) the variation in the length of the cycle. In the present series, for example, the range of cycle lengths extended from twenty-four to thirty-five days. Perhaps because of these obstacles, in none of the previous investigations dealing with gonadotropin excretion was an attempt made to derive a composite pattern from individual cycles.

The first difficulty is readily surmounted by averaging for each subject the day-to-day departure from the geometric mean of her own cycle. The second difficulty is more formidable. If the excretion data be arranged in accordance with the days of the cycle (Fig. 2), the day on which menstruation begins serves as an external point of reference, and optimal summation is achieved for the days in closest proximity to the reference point, *i.e.*, the days of menstruation and the early follicular phase. However, the further one progresses in the composite cycle, the more the pattern is distorted in consequence of the variations in cycle length. The peak period of mid-cycle excretion becomes spuriously prolonged and the summated luteal phase comprises fewer and fewer cycles.

Conversely, if the data be arranged in accordance with the number of days prior to the next menstrual period (Fig. 3) optimal summation is secured for the days of the premenstruum and the mid-luteal phase. Moreover, because the length of the luteal phase varies less than that of the follicular phase, the mid-cycle peak achieves sharper definition. However, as one works backward the summated follicular phase comprises fewer and fewer cycles and becomes progressively more variable.

If the basal body temperature be employed as an independent variable for grouping the excretion data (Fig. 4), a fulcrum for the mid-portion of

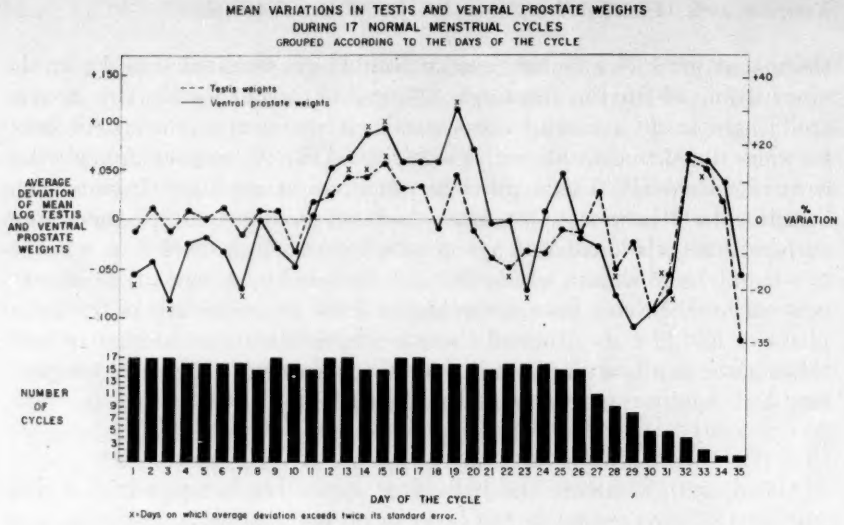
MEAN VARIATIONS IN TESTIS AND VENTRAL PROSTATE WEIGHTS
DURING 17 NORMAL MENSTRUAL CYCLES
GROUPED ACCORDING TO THE DAYS OF THE CYCLE
Testis weights
Ventral prostate weights
AVERAGE DEVIATION OF MEAN LOG TESTIS AND VENTRAL PROSTATE WEIGHTS
% VARIATION
NUMBER OF CYCLES
DAY OF THE CYCLE
x=Days on which average deviation exceeds twice its standard error.

FIG. 2. The average deviations from the mean log testis and ventral prostate weights of each cycle grouped according to the day of the cycle.

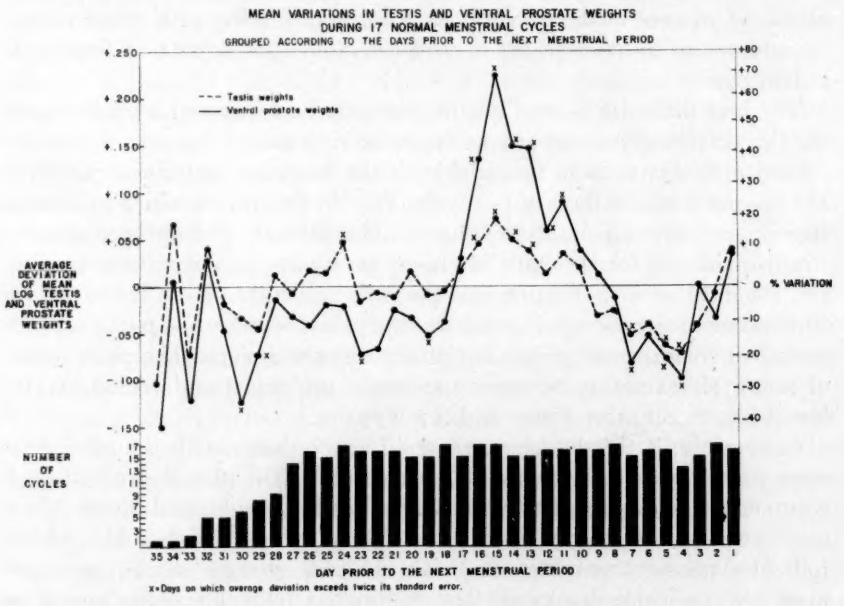
MEAN VARIATIONS IN TESTIS AND VENTRAL PROSTATE WEIGHTS
DURING 17 NORMAL MENSTRUAL CYCLES
GROUPED ACCORDING TO THE DAYS PRIOR TO THE NEXT MENSTRUAL PERIOD
Testis weights
Ventral prostate weights
AVERAGE DEVIATION OF MEAN LOG TESTIS AND VENTRAL PROSTATE WEIGHTS
% VARIATION
NUMBER OF CYCLES
DAY PRIOR TO THE NEXT MENSTRUAL PERIOD
x=Days on which average deviation exceeds twice its standard error.

FIG. 3. The average deviations from the mean log testis and ventral prostate weights of each cycle grouped according to the days prior to the next menstrual period.

In these and in subsequent figures, departures of composite values from the baseline which exceeded twice their standard error were regarded as statistically significant and are marked with an X.

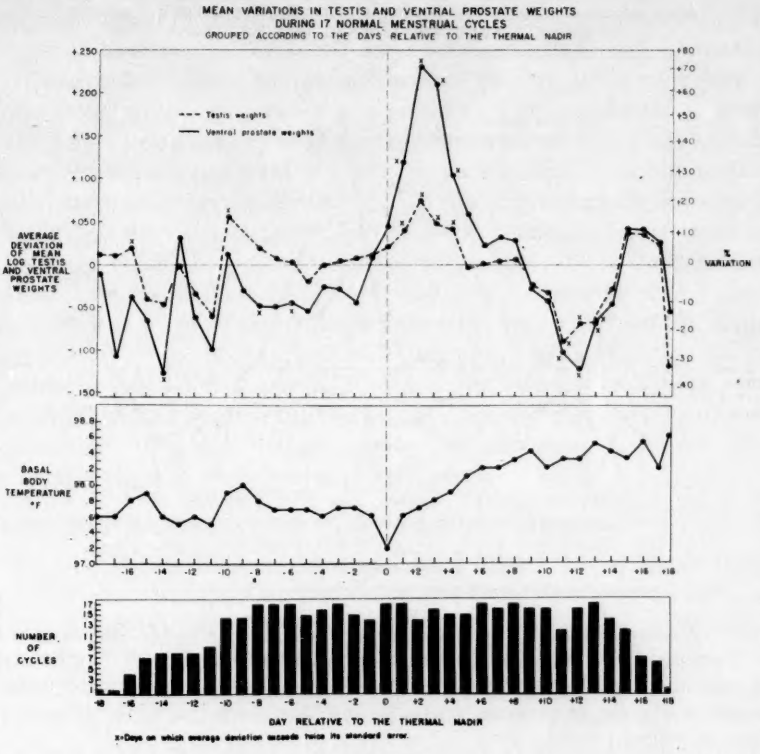
MEAN VARIATIONS IN TESTIS AND VENTRAL PROSTATE WEIGHTS
DURING 17 NORMAL MENSTRUAL CYCLES
GROUPED ACCORDING TO THE DAYS RELATIVE TO THE THERMAL NADIR
Testis weights
Ventral prostate weights
AVERAGE DEVIATION OF MEAN LOG TESTIS AND VENTRAL PROSTATE WEIGHTS
% VARIATION
BASAL BODY TEMPERATURE °F
NUMBER OF CYCLES
DAY RELATIVE TO THE THERMAL NADIR
x=Days on which average deviation exceeds twice its standard error.

FIG. 4. The average deviations from the mean log testis and ventral prostate weights of each cycle grouped according to the day of minimum basal body temperature.

the cycle is acquired. According to this schema, the day on which the basal body temperature reaches its lowest reading during the cycle is taken as the mid-point[4] and is designated as day 0. Days preceding the day of minimum temperature become days -1, -2, etc. and those following, $+1$, $+2$, etc. Optimal summation is thus obtained for the period during which the most pronounced changes in gonadotropin excretion occur. However, the further one departs in either direction from the thermal nadir, the smaller the number of cycles summated and the more variable the result.

A 28-day composite cycle incorporating the data derived by these 3 methods of longitudinal summation is depicted in Figure 5. It is composed

[4] In cycles in which no one minimum temperature reading was observed, the day on which there was recorded the last low temperature before the sustained rise was regarded as the day of minimum temperature.

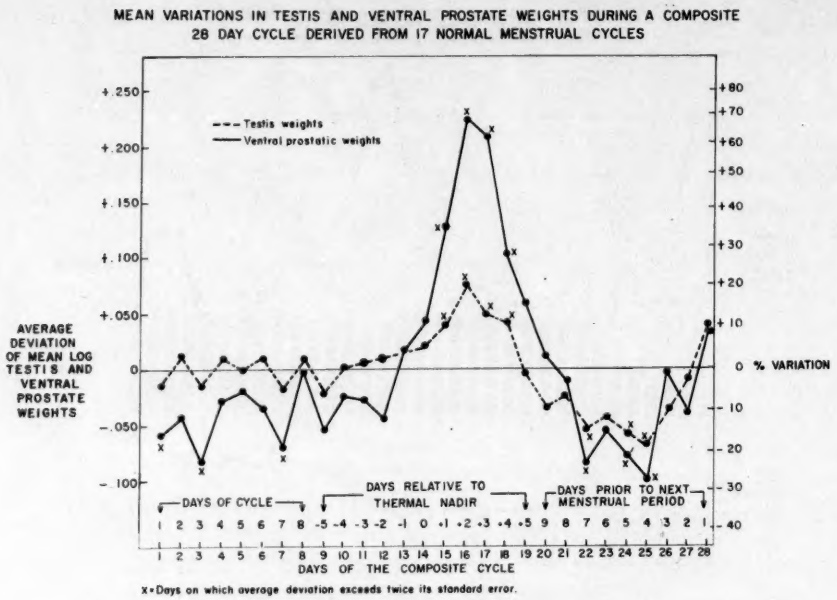
MEAN VARIATIONS IN TESTIS AND VENTRAL PROSTATE WEIGHTS DURING A COMPOSITE 28 DAY CYCLE DERIVED FROM 17 NORMAL MENSTRUAL CYCLES
Testis weights
Ventral prostatic weights
AVERAGE DEVIATION OF MEAN LOG TESTIS AND VENTRAL PROSTATE WEIGHTS
% VARIATION
DAYS OF CYCLE
DAYS RELATIVE TO THERMAL NADIR
DAYS PRIOR TO NEXT MENSTRUAL PERIOD
DAYS OF THE COMPOSITE CYCLE
X = Days on which average deviation exceeds twice its standard error.

FIG. 5. A composite 28-day cycle which represents the average deviations from the mean log testis and ventral prostate weights for each cycle, grouped: (a) in the initial third, according to the day of the cycle; (b) in the middle third, according to the day of minimum basal body temperature; (c) in the final third, according to the days prior to the next menstrual period.

of 3 approximately equal parts corresponding to the summation according to the days of the cycle, the days preceding and following the day of minimum basal body temperature and the days prior to the next menstrual period. A striking parallelism obtains between the changes in the weights of the ventral prostate and testis. It would appear that the cycle is characterized by: 1) erratic and (with 1 exception) insignificant fluctuations in both FSH and ICSH excretion during the follicular phase of the cycle; 2) an increase in the level of excretion of both hormones which begins and may possess biologic significance before the thermal nadir, the levels attaining statistical significance on the day after the thermal nadir and remaining appreciably above the baseline for a total of four days; 3) a progressive trend downward, which reaches significantly low levels toward the close of the luteal phase; and 4) a subsequent trend upward during the premenstruum, which fails to attain statistical significance.

The only composite curves available for comparison are those of Buchholz (30), who summated his data biologically by combining the urine

specimens collected by 17 normal women throughout the menstrual cycle into serial 48-hour pools. The ICSH and "total gonadotropin" content of the pools were determined by the rat prostate and mouse uterine weight methods, respectively, the extract of a grand pool prepared from aliquots of all the urines being employed as a reference standard. The 2 biologically summated curves are remarkably similar to each other and to the mathematically summated curves derived in this study, particularly as regards the timing and duration of the mid-cycle peak. Buchholz found that the only pools in which the potency differed appreciably from the standard were those comprising days 13 plus 14, and days 15 plus 16 of this composite cycle. The large measure of agreement between the 2 studies seems ascribable, in part, to the comparative constancy of cycle length in Buchholz' subjects. Although the cycle of one of his subjects was twenty-six days in length and of another thirty-two days, these variations presumably introduced but little disturbance because the remaining 15 cycles were either twenty-eight or twenty-nine days long.

C. *Quantitative relations between FSH and ICSH excretion*

The quantitative relations between the day-to-day variations in testis and ventral prostate weight were examined by computing regression equations expressing the change in prostate weight per unit of logarithmic change in testis weight. Obviously, biologic as well as statistical considerations enter into the interpretation of such regression coefficients, since the ICSH present in an unfractionated extract increases testis weight to some extent. Steep slopes indicating a preponderance of ICSH might be expected to be reduced below their true value by the presence of FSH; gentle slopes indicating a preponderance of FSH might be expected to be increased by the presence of ICSH.

If such calculations be applied to the individual cycles, the mean regression coefficient for days 0 to +4 inclusive is 1.769 as against 0.978 for the other days of the cycle. Although the difference is statistically significant ($P<0.05$), it was felt that, in view of the brevity of ovulation, an examination of the day-by-day changes in the composite cycle might be more revealing. Therefore, coefficients expressing the regression of ventral prostate upon testis weight were computed[5] for each day of the 28-day composite cycle. They are depicted in Figure 6. It may be seen that during mid-

[5] The standard error of each coefficient was also computed and the squares of these standard errors were averaged, using as weights the number of cycles entering into the calculation of each coefficient. Regression coefficients departing by more than twice this average $S.E._b$ from the cycle mean of 1.153 were regarded as statistically significant ($P<0.05$).

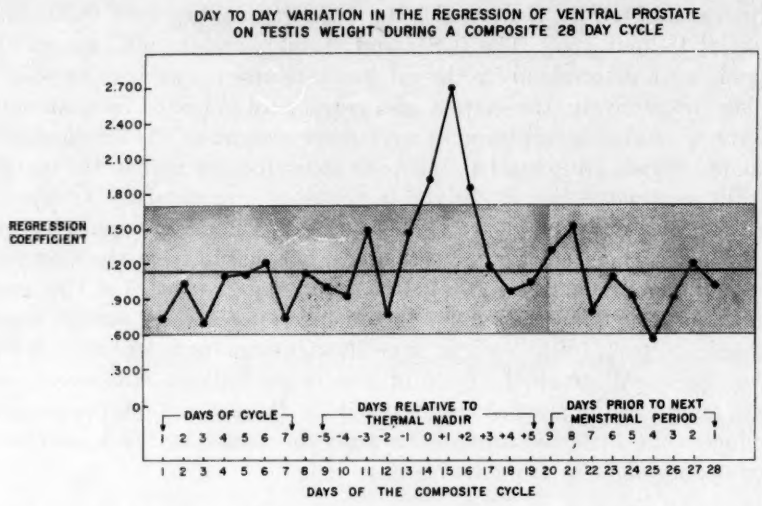
DAY TO DAY VARIATION IN THE REGRESSION OF VENTRAL PROSTATE ON TESTIS WEIGHT DURING A COMPOSITE 28 DAY CYCLE
REGRESSION COEFFICIENT
2.700
2.400
2.100
1.800
1.500
1.200
.900
.600
.300
0
DAYS OF CYCLE
DAYS RELATIVE TO THERMAL NADIR
DAYS PRIOR TO NEXT MENSTRUAL PERIOD
1 2 3 4 5 6 7 8 -5 -4 -3 -2 -1 0 +1 +2 +3 +4 +5 9 8 7 6 5 4 3 2 1
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28
DAYS OF THE COMPOSITE CYCLE

FIG. 6. The day-to-day variation in the coefficient expressing the regression of ventral prostate on testis weight throughout the 28-day composite cycle depicted in Figure 5. The shaded area represents twice the average standard error about the mean trend. Departures from the mean by more than twice the standard error were regarded as statistically significant.

cycle on days 0, +1 and +2 the slopes significantly exceeded the cycle mean.

Data of other investigators who have recorded testis and prostate weights in studies employing the hypophysectomized male rat for the assay of unfractionated pituitary gonadotropic extracts and of FSH and ICSH preparations of varying degrees of purity have been similarly analyzed. As shown in Table 3, the slope of the regression line of ventral prostate on testis weight for the unfractionated pituitary extracts is similar to that recorded for unfractionated urinary extracts except during mid-cycle. Immediately prior to and during the shift in basal body temperature, the slope for the urinary extracts increases significantly and approximates that obtained with pituitary ICSH preparations.

The interpretation of results obtained with unfractionated extracts is difficult. However, these data suggest that during mid-cycle there may be a change in the ratio of ICSH to FSH, and a transient predominance of ICSH. Support for such an interpretation is provided by Simpson *et al.* (34), who determined the FSH and ICSH potency of the pituitaries of normal adult female monkeys killed on different days of the menstrual cycle. Only general comparisons can be made between the results of Simpson's study and the present study, since follicle stimulation and interstitial-cell

TABLE 3. SLOPES OF THE REGRESSION LINE OF VENTRAL PROSTATE ON TESTIS WEIGHT OBTAINED BY INVESTIGATORS EMPLOYING THE HYPOPHYSECTOMIZED MALE RAT FOR THE ASSAY OF PITUITARY GONADOTROPIC PREPARATIONS

Material	Animal source	Author	Regression coefficient of the ventral prostate weight on testis weight (in logarithms)
Unfractionated gonadotropic extract	Swine	Greep *et al.* (1)	(a) 0.991 (b) 1.082
FSH	Swine	Greep *et al.* (1)	0.166
		Greep *et al.* (31)	0.221
	Sheep	McShan *et al.* (32)	0.295
ICSH	Swine	Greep *et al.* (1)	1.517
	Swine	Shedlovsky *et al.* (3)	3.020
	Sheep	Simpson *et al.* (33)	1.952 (L_{1063}TP)
			2.620 (L_{1066}TP)
			3.301 (L_{1087}BS)
			2.312 (L_{1120}B)
			1.600 (L_{1137}CP)

repair in the ovaries of the hypophysectomized female rat were employed by the former authors as the criteria of FSH and ICSH effects. However, it is of interest that Simpson found that both FSH and ICSH attained maximal levels in mid-cycle. The pituitary content of ICSH increased relatively more, so that the ratio of ICSH to FSH rose from its usual value of 3 to 1 to a value of 10 to 1 on days 11 and 15.

COMMENT

Both individual and composite curves appear to contribute valid information, although neither is capable of revealing the "whole truth" because of the inherent vagaries of bioassay, the insensitivity of the methods employed and the dilution error introduced by pooling. Consequently, we are inclined to accord considerable weight to apparently clear-cut features of either individual or composite curves.

These data are susceptible to a variety of interpretations. Expressed in the traditional dualistic terminology, they suggest that the normal menstrual cycle may be characterized by: 1) A mid-cycle peak period of FSH and ICSH excretion, during the early phase of which there is a change in the ratio of ICSH to FSH and a transient preponderance of ICSH. The onset of the mid-cycle peak is closely related in time to the shift in basal body temperature. 2) A late luteal trough followed by a rising trend in the

excretion of both hormones, beginning toward the end of the luteal phase and continuing into menstruation. Obviously, during many cycles substantial amounts of FSH are released at other times as well.

Acknowledgment

We wish to express our appreciation to the Misses Jean Richards and Elizabeth Wilder and to Mrs. Margaret Proctor and Mrs. Marianne Purdy for their expert technical assistance and to the Misses Jean Richards and Pamela Malcolm for their help in preparing the data for statistical analysis.

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THE URINARY EXCRETION OF INTERSTITIAL-CELL AND FOLLICLE-STIMULATING HORMONE ACTIVITY BY WOMEN WITH DISEASES OF THE REPRODUCTIVE SYSTEM*

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ABSTRACT

Changes in the day-to-day excretion of ICSH have been determined qualitatively over periods of six to 122 days in 11 young women with diseases of the reproductive tract by means of a technique which depends upon the repair of ventral prostatic atrophy in the hypophysectomized immature male rat. Changes in the excretion of FSH were estimated from concomitant changes in in testicular weight. The quantitative relations between the day-to-day variations in rat ventral prostate and testis weight were examined by computing regression equations expressing the logarithmic change in prostatic weight per unit of change in testicular weight.

It was found that: 1) Recognizable differences existed between these patients and normally menstruating women with respect to the excretion levels of both ICSH and FSH activity. 2) Erratic fluctuations were characteristic of the individual patterns of ICSH and FSH excretion in all of the subjects. Only in the 2 patients with the Stein-Leventhal syndrome who ovulated during postoperative studies was a mid-cycle peak discernible. 3) In 6 patients the regression coefficients fell significantly below the normal mean, suggesting a lowering of ICSH relative to FSH excretion.

"SPOT" determinations of the level of urinary gonadotropin excretion, introduced into clinical medicine by Albright *et al.* (1, 2), have become a valued tool in endocrinologic diagnosis. Multiple-dose assays, performed by testing serial dilutions of an extract prepared from one or at most two 24-hour specimens of urine, ordinarily permit the differentiation

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† Requests for reprints should be sent to the Vincent Memorial Hospital, Boston, Massachusetts.

of gonadal from pituitary failure. However, in less extreme disturbances of reproductive function, the information contributed by spot gonadotropin assays is of limited usefulness. In part this is due to a lack of information concerning the pattern of gonadotropin excretion from day to day.

Though serial determinations might be thought to be a logical, and indeed necessary, complement to spot assays they have only rarely been performed, except during the normal menstrual cycle. Frank *et al.* (3) distinguished between 4 amenorrheic patients whose gonadotropin excretion remained continuously high over a period of one or more months, and 6 whose urine contained no demonstrable gonadotropin. Pedersen-Bjergaard and Tønnesen (4) found that the gonadotropin excretion patterns of 2 patients with secondary amenorrhea differed but little from those of normally menstruating women. Albert *et al.* (5) recently demonstrated that although the gonadotropin titer is characteristically high in postmenopausal women, the level of excretion undergoes at least a twofold fluctuation from day to day.

The purpose of this communication is to present a qualitative description of the pattern of urinary interstitial-cell-stimulating hormone (ICSH) excretion in 11 women with diseases of the reproductive tract, and to compare the results with those reported (6) in normal adult women. The data were obtained by means of a technique for identifying pituitary ICSH activity which depends upon the repair of ventral prostatic atrophy in the hypophysectomized immature male rat (7). The weights of the rat testes were also recorded as an index[1] of follicle-stimulating hormone (FSH) activity. Although the results can be interpreted in a number of different ways, we have elected to retain the original dualistic nomenclature which implies that FSH exists as an entity distinct from ICSH.

METHODS

The patients were young women, 7 of whom suffered from menorrhagia and oligomenorrhea and 4 of whom were completely amenorrheic. The clinical features of their cases are summarized in Table 1. The urine was collected in serial 24-hour specimens over periods ranging from six to 122 days. Creatinine determinations were performed as a check against gross errors of collection. The basal body temperature was recorded daily.

Since it was desired to compare the excretion patterns in the patients with those previously obtained in normal women, a single-dose assay of individual specimens was preferred over a multiple-dose assay of pooled specimens. An 18-hour aliquot was concentrated by means of kaolin adsorption and the equivalent of six hours of urine was injected into each of 3 rats according to a method previously described (6). Prior to statistical analysis the data were converted to logarithmic form.

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[1] The data which appear to justify the qualified use of the weight of the testis for this purpose have been summarized elsewhere (6).

TABLE 1. DESCRIPTION OF PATIENTS WITH DISORDERS OF REPRODUCTIVE FUNCTION

Patient			Diagnosis	Comment	Gonadotropin excretion, mouse uterine weight method* (units per 24 hrs.)	
Name	Age (yrs.)	Marital status			Greater than	Less than
				A. *Amenorrhea or oligomenorrhea*		
J.F.	15	S	Functional bleeding	Ovulatory menstruation after cyclic hormone therapy.	—	—
M.R.	29	M	Functional bleeding, ? psychogenic	Ovaries normal except for absence of corpora lutea at incidental appendectomy.	—	13
A.B.	25	M	? Stein-Leventhal syndrome	Classical history and physical findings, not yet confirmed by operation.	13	52
D.E.	25	M	Stein-Leventhal syndrome	Ovulatory menstruation and pregnancy after subsequent wedge resection of the ovaries.	13	52
E.K.	29	M	Stein-Leventhal syndrome	Ovulatory menstruation and pregnancy after subsequent wedge resection of the ovaries.	13	52
M.B.	26	S	Stein-Leventhal syndrome	Ovulatory menstruation after subsequent wedge resection of the ovaries.	13	52
V.H.	26	M	Stein-Leventhal syndrome	Infrequent ovulatory menstruation without pregnancy after previous wedge resection of the ovaries.	13	52
				B. *Amenorrhea*		
W.F.	24	S	"Boarding-school" amenorrhea	Ovulatory menstruation during vacations at home.	—	6.5
V.K.	27	S	Psychogenic amenorrhea	Severe neurotic character defect.	6.5	13
M.Q.	26	S	Hypopituitarism	Enlarged sella turcica, thought to be due to a chromophobe adenoma of the pituitary.	6.5	13
R.A.	31	M	Rudimentary ovaries	Small ovaries containing a few scattered primordial follicles found at laparotomy.	(a) 13 (b) 52	52 104

* Normally positive for greater than 13 and less than 52 mouse units (specimens accidentally collected in mid-cycle occasionally positive for 52 mouse units in normal women).

The remaining six-hour aliquot of the 24-hour specimen was reserved in order that determinations of the pregnanediol content (8) might be made at intervals. In several of the patients who menstruated occasionally, an endometrial biopsy specimen was secured during the first twenty-four hours of flow. One patient with the Stein-Leventhal syndrome (V.H.), who was studied for four months postoperatively, proved to have a secretory endometrium at the end of the collection period. In another patient with this syndrome (M.B.), although endometrial biopsy was not performed, ovulation was presumed to have occurred during the postoperative studies because of the biphasic character of the temperature pattern and the presence of pregnanediol in the urine. All other biopsies revealed a proliferative endometrium. This fact, coupled with the uniformly monophasic basal body temperature curves and the absence of pregnanediol from the urine, was taken to indicate that, in all other instances, ovulation did not take place during the collection periods.

In 10 of the 11 patients, spot determinations of urinary gonadotropin excretion were also made in Dr. Fuller Albright's laboratory, by means of the mouse uterine weight method. The results are included in Table 1.

RESULTS

A. *Composite values*

In Table 2 are summarized the geometric mean weights of the rat testis and prostate for the entire series of specimens studied in each patient, together with the composite mean values for normally menstruating women; also listed are the testis and prostate weights in the untreated control animals. It is evident that the patients differed significantly from normal women with respect to the excretion levels of both ICSH and FSH activity.

In the 5 patients with functional bleeding, oligomenorrhea and amenorrhea due to hypopituitarism or psychologic disturbances, the ICSH excretion was markedly depressed; in the patient with rudimentary ovaries and in all 4 of the patients with untreated Stein-Leventhal syndrome, the excretion of ICSH was elevated. Only in the 2 Stein-Leventhal patients who were studied postoperatively did the excretion of ICSH approach normal levels. Significant alterations in the excretion of FSH activity accompanied the changes in ICSH activity, except in 3 instances (J.F., D.E. and M.B., postoperatively).

B. *Individual excretion patterns*

The excretion patterns in the 9 patients who were studied for comparatively long periods are depicted in Figures 1–9. In those patients whose gonadotropin excretion was depressed, whether adjudged by rat testis or prostate weights, the excretion patterns resembled one another rather closely. Although fluctations were evident, none was pronounced, and on many days the titers of hormone fell below the detectable level. In the lack of anything resembling a mid-cycle peak these curves are reminiscent

TABLE 2. THE MEAN LOG VENTRAL PROSTATE AND TESTIS WEIGHTS, WITH THEIR RESPECTIVE STANDARD DEVIATIONS, OF RATS RECEIVING INJECTIONS OF EXTRACTS OF URINE FROM PATIENTS WITH REPRODUCTIVE DISORDERS

Those geometric mean weights (antilogarithms) exhibiting a statistically significant difference ($P < 0.05$) from the geometric mean prostatic and testicular weights of rats receiving injections of extracts of urine from normal women are italicized

Patient	Diagnosis	No. of consecutive days studied	Mean log v. prost. wt.	S.D.*	Geom. mean v. prost. wt. (antilog MLVPW) (mg.)	Mean log testis wt.	S.D.	Geom. mean testis wt. (antilog MLTW) (mg.)
			A. *Menorrhagia or oligomenorrhea*					
J.F.	Functional bleeding	25	0.980	0.054	*9.6*	2.348	0.047	222.8
M.R.	Oligomenorrhea, sterility	27	0.946	0.060	*8.8*	2.339	0.069	*218.3*
		44	0.930	0.129	*8.5*	2.331	0.071	*214.3*
A.B.	? Stein-Leventhal syndrome	30	1.319	0.138	*20.8*	2.470	0.062	*295.1*
D.E.	Stein-Leventhal syndrome (preop.)	28	1.308	0.142	*20.3*	2.401	0.063	251.8
E.K.	Stein-Leventhal syndrome (preop.)	36	1.344	0.120	*22.1*	2.413	0.066	*258.8*
M.B.	Stein-Leventhal syndrome (preop.)	30	1.370	0.139	*23.4*	2.483	0.055	*304.1*
	(postop.)	35	1.204	0.150	*16.0*	2.372	0.098	235.5
V.H.	Stein-Leventhal syndrome (postop.)	122	1.072	0.162	*11.8*	2.356	0.090	*227.0*
			B. *Amenorrhea*					
W.F.	"Boarding-school" amenorrhea	28	0.954	0.048	*9.0*	2.355	0.064	*226.5*
V.K.	Psychogenic amenorrhea	7	0.821	0.048	*6.6*	2.252	0.086	*178.6*
M.Q.	Hypopituitarism	6	0.859	0.063	*7.2*	2.270	0.030	*186.3*
R.A.	Rudimentary ovaries	27	1.363	0.057	*23.1*	2.553	0.036	*357.3*
13 normal women throughout 17 menstrual cycles		463	1.139	0.157	13.8	2.388	0.094	244.3
Untreated control rats		—	0.858	0.110	7.2	2.172	0.062	148.6

of those which D'Amour (9, 10) observed occasionally in normally menstruating women and which he regarded as indicative of anovulation.

There was also a measure of resemblance between the patterns in those patients whose excretion levels were significantly elevated. Erratic fluctuations were characteristic of all. Only in the patterns of the 2 patients who ovulated during postoperative studies was a mid-cycle peak discernible.

From the configuration of the excretion patterns one might predict that good agreement should obtain between the results of serial and spot determinations in those patients who consistently excreted barely detectable amounts of hormone. In those who excreted elevated but fluctuating amounts only chance agreement might be anticpated.

It would appear that such predictions were in large measure fulfilled

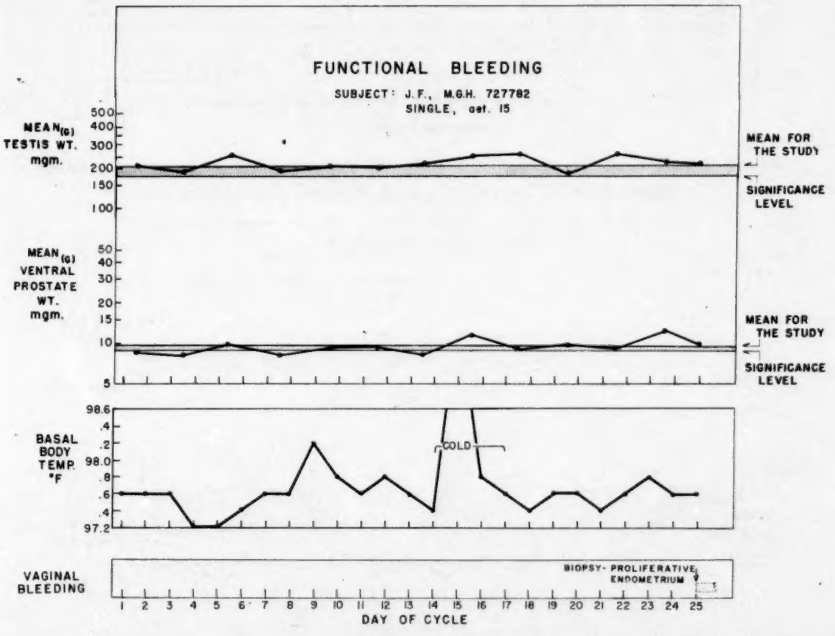

FIG. 1. Changes in rat testis and ventral prostate weights induced by urinary extracts from Subject J.F., a 15-year-old schoolgirl. In this and all subsequent figures the geometric mean weights are plotted on a logarithmic scale. The "significance level" indicates that geometric mean weight at or above which the organ weights of the test animals (based on data from 3 rats) may be regarded as differing significantly ($P = 0.025$) from those of the control animals. The level was adjusted slightly from year to year in accordance with the organ weights of the control animals during the year in which the particular assays were performed.

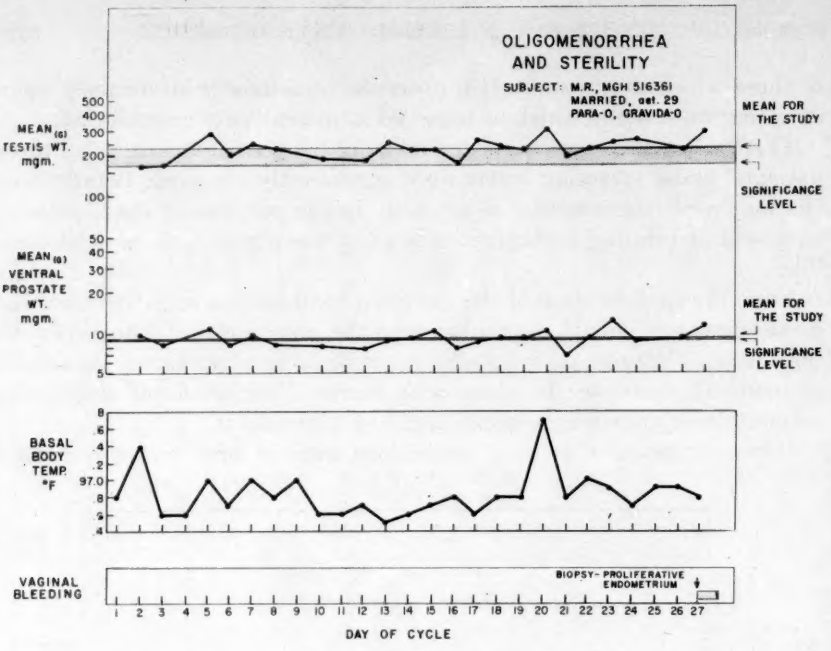

FIG. 2. Changes in rat testis and ventral prostate weights induced by urinary extracts from Subject M.R., a 29-year-old married woman.

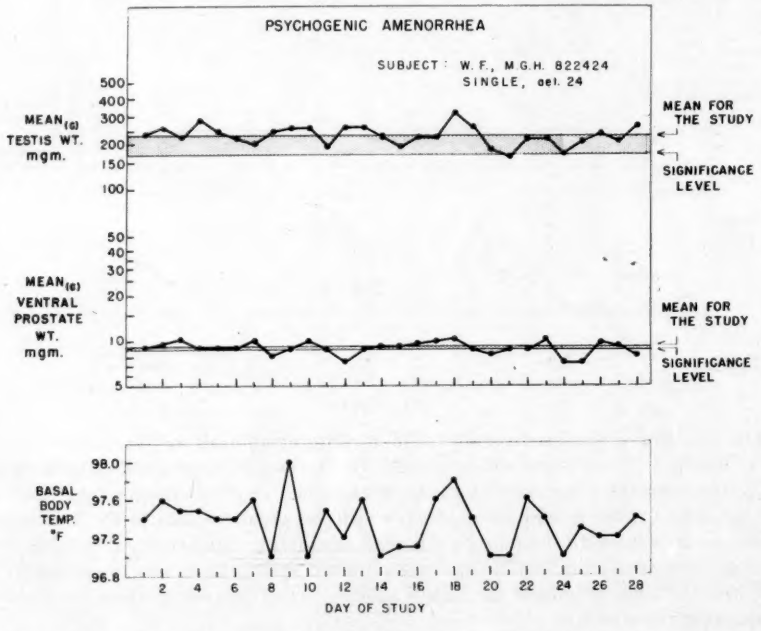

FIG. 3. Changes in rat testis and ventral prostate weights induced by urinary extracts from Subject W.F., a 24-year-old unmarried hospital dietitian.

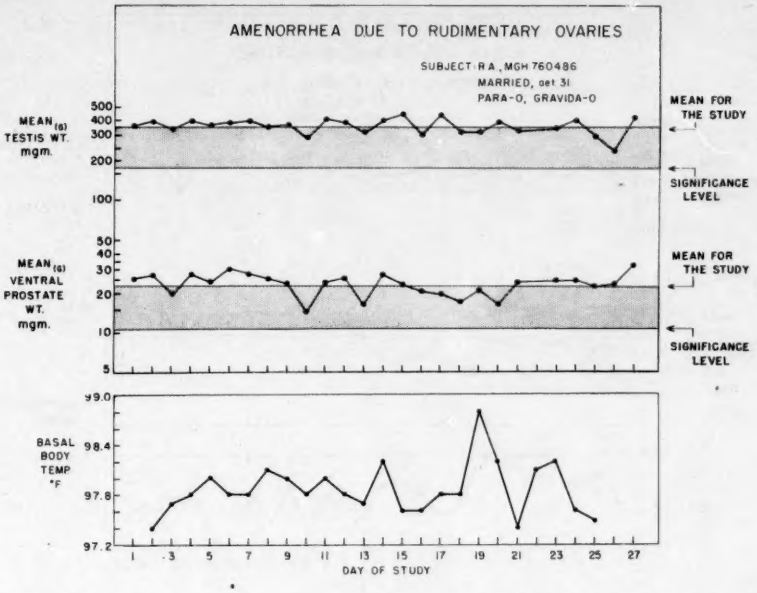

FIG. 4. Changes in rat testis and ventral prostate weights induced by urinary extracts from Subject R.A., a 31-year-old married woman.

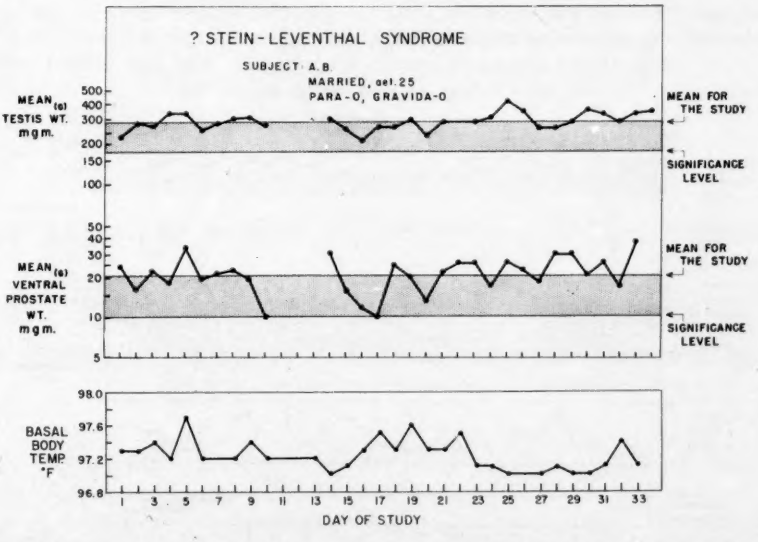

FIG. 5. Changes in rat testis and ventral prostate weights induced by urinary extracts from Subject A.B., a 25-year-old married woman with sterility and classical findings of the Stein-Leventhal syndrome, not yet confirmed by operation.

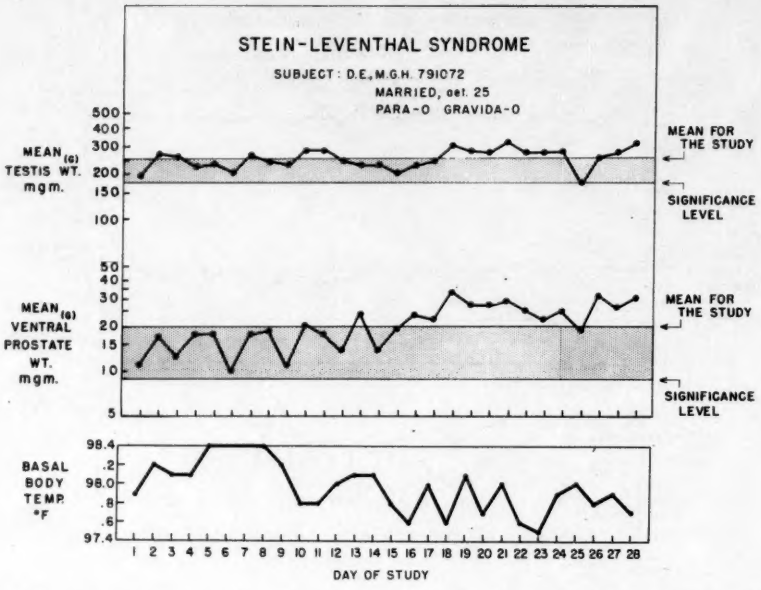

FIG. 6. Changes in rat testis and ventral prostate weights induced by urinary extracts obtained preoperatively from Subject D.E., a 25-year-old married woman with the Stein-Leventhal syndrome. She conceived within two months after wedge resection of the ovaries and was delivered of a normal infant.

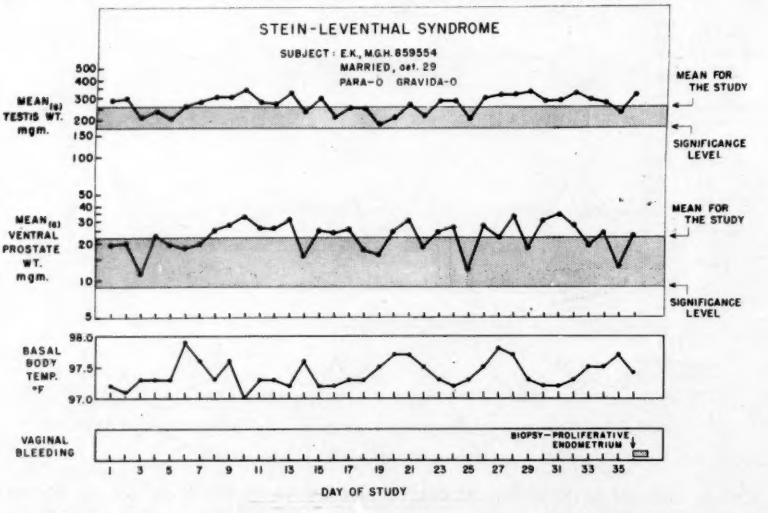

FIG. 7. Changes in rat testis and ventral prostate weights induced by urinary extracts obtained preoperatively from Subject E.K., a 29-year-old married woman with the Stein-Leventhal syndrome. She conceived two years after wedge resection of the ovaries and was delivered of a normal infant.

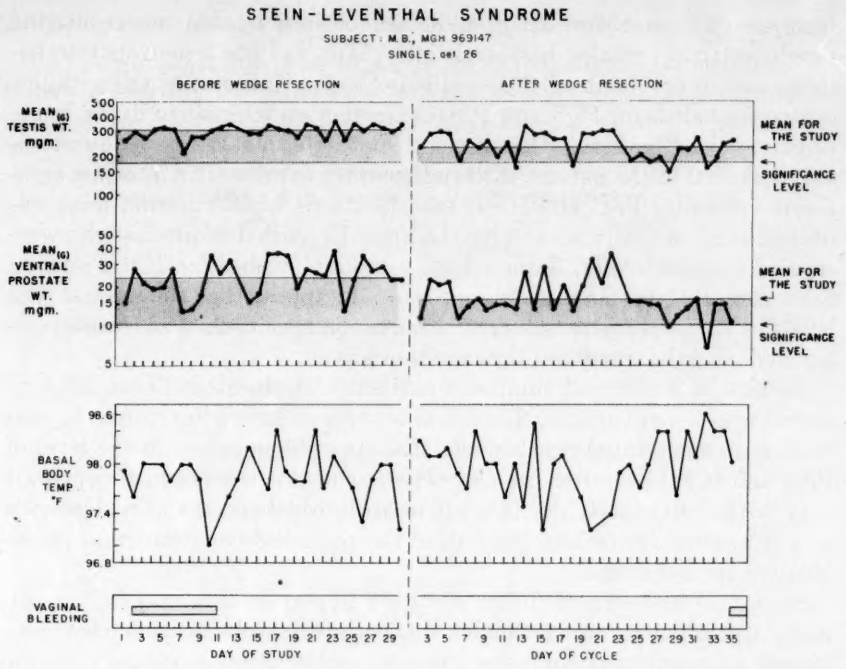

FIG. 8. Changes in rat testis and ventral prostate weights induced by urinary extracts obtained from Subject M.B., a 26-year-old unmarried secretary with the Stein-Leventhal syndrome. During the portion of the study conducted after wedge resection, pregnanediol was demonstrable in the urine premenstrually.

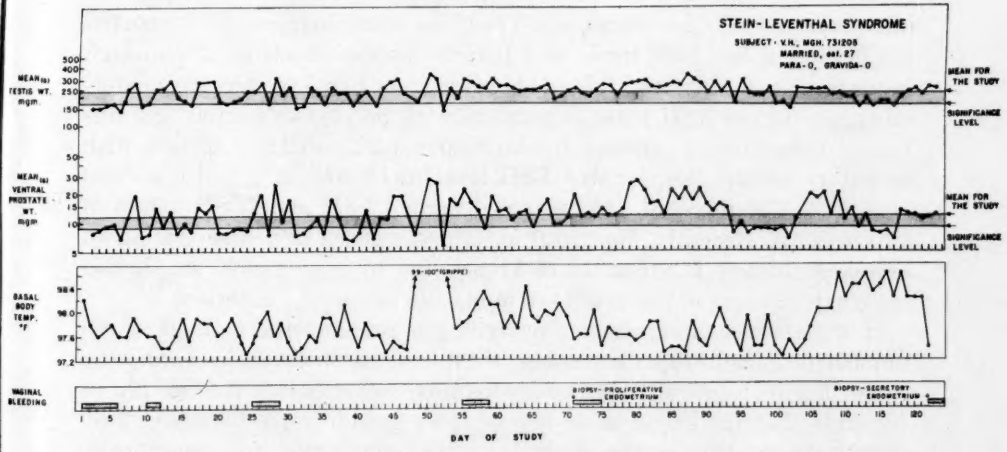

FIG. 9. Changes in rat testis and ventral prostate weights induced by urinary extracts obtained post-operatively from Subject V.K., a 29-year-old married woman with the Stein-Leventhal syndrome. Toward the close of the study she ovulated and menstruated from a secretory endometrium.

(compare Tables 1 and 2). Spot determinations by the mouse uterine weight method revealed low levels in 1 (M.R.) of the 2 metropathic patients and in the 3 amenorrheic patients (W.F., V.K. and M.Q.) with low composite values for FSH and ICSH excretion as determined in the hypophysectomized male rat. One of the 2 spot determinations was questionably elevated in the patient with rudimentary ovaries (R.A.), whose composite values for FSH and ICSH excretion were high. The spot level was normal in all of the patients with the Stein-Leventhal syndrome who were studied preoperatively, despite high composite values for ICSH and, in most instances, for FSH excretion. It would appear that the normal spot levels in the latter patients were obtained on specimens accidentally collected during the troughs of the excretion waves.

Because of the limited number of patients with the Stein-Leventhal syndrome who were available, the results of these serial studies cannot be said to have demonstrated conclusively that sporadic increases in the level of FSH and ICSH excretion are characteristic of this condition. However, it may be that ultimately this fact will be established and that it will provide an explanation for certain features of the pathologic anatomy and physiology of the syndrome.

Stein (11) has suggested that pituitary hypersecretion may be the primary disturbance, citing Zondek's experimental observation that prolonged gonadotropic stimulation of the ovaries leads eventually to the production of multiple follicle cysts, a thickened capsule and fibrosis of the ovarian stroma. However, little support for Stein's hypothesis has been provided by the majority of investigators who have studied urinary gonadotropin excretion by means of spot assays. Schneider *et al.* (12) obtained elevated FSH levels in only 3 of 10 patients; Ingersoll (13) reported the finding of low FSH levels in 1 patient, normal levels in 22 and high levels in only 3. Haas and Riley (14) made from 1 to 5 preoperative determinations of the FSH level in patients with polycystic ovaries and disturbed menstruation; among 6 who responded to wedge resection with ovulatory menses, an elevated FSH level was found in 1 and a normal level in 5. Cervino *et al.* (15) obtained normal FSH and ICSH values in two patients. Recently, Keettel *et al.* (16) succeeded in demonstrating an excess of urinary ICSH in 10 of 11 patients by spot assays, employing histologic changes in the ovaries of immature rats as the criterion.

In the absence of agreement concerning so fundamental an issue as the number of gonadotropic hormones, these various observations cannot be reconciled in an entirely satisfactory manner. Nevertheless, it seems fair to conclude that the excretion of one or more gonadotropic hormones fluctuates abnormally in the Stein-Leventhal syndrome and periodically reaches high levels.

C. *Quantitative relations between FSH and ICSH excretion*

The quantitative relations between the day-to-day variations in testis and ventral prostate weights have been examined by computing regression equations expressing the logarithmic change in prostate weight per unit of

TABLE 3. COEFFICIENTS EXPRESSING THE REGRESSION OF VENTRAL PROSTATE ON TESTIS WEIGHT (IN LOGARITHMS) THROUGHOUT THE URINE COLLECTION PERIODS IN PATIENTS WITH REPRODUCTIVE DISORDERS

Patient	Diagnosis	No. of consecutive days studied	Regression coefficient*
	A. *Menorrhagia or oligomenorrhea*		
J.F.	Functional bleeding	25	*0.581*
M.R.	Oligomenorrhea, sterility	27	*0.534*
		44	0.746
A.B.	? Stein-Leventhal syndrome	30	1.056
D.E.	Stein-Leventhal syndrome (preop.)	28	1.267
E.K.	Stein-Leventhal syndrome (preop.)	36	1.217
M.B.	Stein-Leventhal syndrome (preop.)	30	1.585
	(postop.)	35	1.082
V.H.	Stein-Leventhal syndrome (postop.)	122	1.428
	B. *Amenorrhea*		
W.F.	"Boarding-school" amenorrhea	28	*0.239*
V.K.	Psychogenic amenorrhea	7	*0.012*
M.Q.	Hypopituitarism	6	*−0.357*
R.A.	Rudimentary ovaries	27	*0.637*
Normal adult women		463	1.198 ± 0.162

* Regression coefficients differing significantly ($P < 0.01$) from the composite trend in normal adult women are italicized.

change in testis weight[2] (Table 3). In many patients the slopes exhibited no material difference from the composite value for the normal cycle, which approximates that obtained with unfractionated pituitary extracts (6). In no instance did the slope significantly exceed the normal mean.

The 6 patients whose regression coefficients fell significantly below the normal mean are divisible into a group comprising only Patient R.A., in whom the composite level of FSH and ICSH excretion was clearly elevated, and a group of 5 (Patients J.F., M.R., W.F., V.K. and M.Q.) in whom the composite level of excretion of one or both hormones was significantly depressed. In Patient R.A., it would appear that the elevation of ICSH excretion was less than that of FSH excretion. In the 5-patient group the depression of ICSH excretion was greater than that of FSH excretion.

In suggesting that the level of ICSH excretion is low relative to FSH excretion, the slopes of the latter group recall the observations of Brown (17) and of Fletcher and Brown (18). These investigators obtained a low value for total gonadotropin relative to FSH excretion in certain amenorrheic women whose urine was subjected to simultaneous assay by means of mouse uterine weight and HCG-augmented ovarian weight methods, respectively.

COMMENT

The results of these studies suggest that between the extremes represented by gonadal and pituitary failure there lie intermediate conditions, such as the Stein-Leventhal syndrome, characterized by detectable abnormalities of lesser magnitude. An ideal description of these states would require serial studies comprising multiple-dose assays against a standard preparation. Laborious and costly though such an undertaking would be, it appears essential if the full clinical potentialities of gonadotropin assay are to be realized.

Acknowledgment

We wish to express our appreciation to the Misses Jean Richards, Elizabeth Wilder and Pamela Malcolm and to Mrs. Margaret Proctor and Mrs. Marianne Purdy for their expert technical assistance, and to Miss Jean Richards for her help in preparing the data for statistical analysis.

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STUDIES ON THE BIOLOGIC CHARACTERIZATION OF HUMAN GONADOTROPINS. III. COMPARISON WITH SHEEP FOLLICLE-STIMULATING HORMONE*

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ABSTRACT

The essential biologic nature of human pituitary gonadotropin as found in urine (HPG) was further investigated by comparison with sheep follicle-stimulating hormone (FSH) by means of assays employing the simultaneous response of the uterus and ovary. The parameters of these assays showed that the ovarian response to HPG was three times that to FSH, and the uterine response to HPG was two and a half times that to FSH. The dose ratios showed that HPG was twice as effective as FSH in inducing secretion of estrogen. The ovarian response to both FSH and HPG was a linear function of the log of the dose; the uterine response to HPG also was a linear function of the log of the dose but the response to FSH was not. It was concluded that HPG is not identical with FSH and that the widespread use in clinical literature of the term "urinary FSH" for an assay of HPG is incorrect.

A PREVIOUS report (1) has shown that human pituitary gonadotropin (HPG, urine) had certain distinguishing characteristics when measured by the simultaneous response of the uterus and ovary of the intact rat. The 6 constants derived from this method of assay were the same for HPG prepared from the urine of men and of postmenopausal or castrated women. In another report (2), the parameters for the assay of human chorionic gonadotropin (HCG, urine) were found to be much different from those of HPG when the same assay system was used. In a dose range of 0.1 to 10 international units of HCG, the ovarian response (like that to HPG) was a linear function of the log of the dose but the uterine response (unlike that to HPG) was biphasic when plotted on semilog paper. Indeed, the uterine response to HCG was an autocatalytic type of curve, whereas that to HPG was a linear function of the log of the dose. In addition, all other constants of the assay of HCG were different from those of HPG. The present report defines the constants of the assay of sheep follicle-stimulating hormone (FSH).

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MATERIAL AND METHODS

The preparation used was kindly supplied by Drs. A. Wilhelmi and S. Ellis, Emory University School of Medicine, Emory University, Georgia. The assay system employed was identical to that used for HPG and HCG, a feature which is obligatory in studies of this kind.

RESULTS

Three assays of FSH are seen in Figures 1, 2 and 3. Figure 1 shows a 7-point, range-finding assay. The incomplete data in this assay indicated that both the uterine and ovarian responses were linear functions of the log of the dose. On the basis of this curve, another 7-point assay was performed with more judicious spacing of the doses (Fig. 2). The ovarian response again was shown to be a linear function of the log of the dose, but the uterine response was a hyperbolic curve. Confirmation of the latter finding was obtained by the curve shown in Figure 3, a 13-point assay. Little doubt exists that the ovarian response is a linear function of the log of the dose and that the uterine response is hyperbolic. However, the uterine response plots fairly well as a linear function of the log of the dose and was handled as such for reasons given previously.

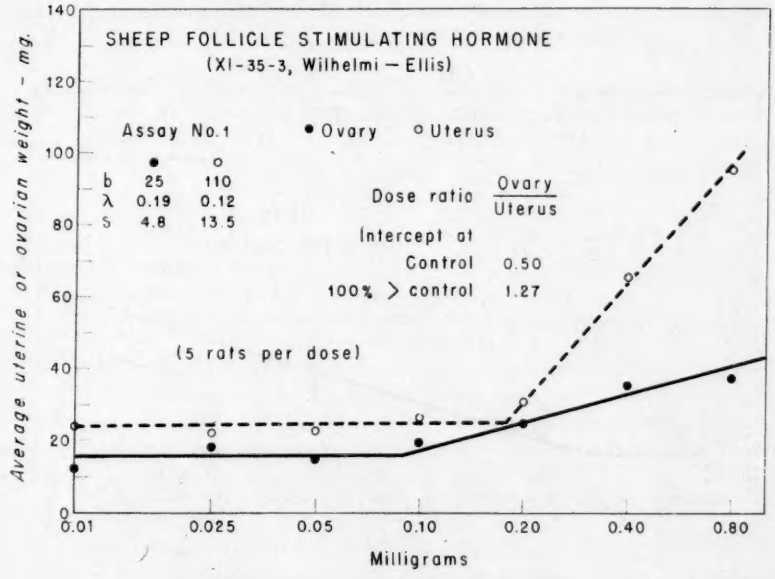

FIG. 1. Seven-point assay of FSH.

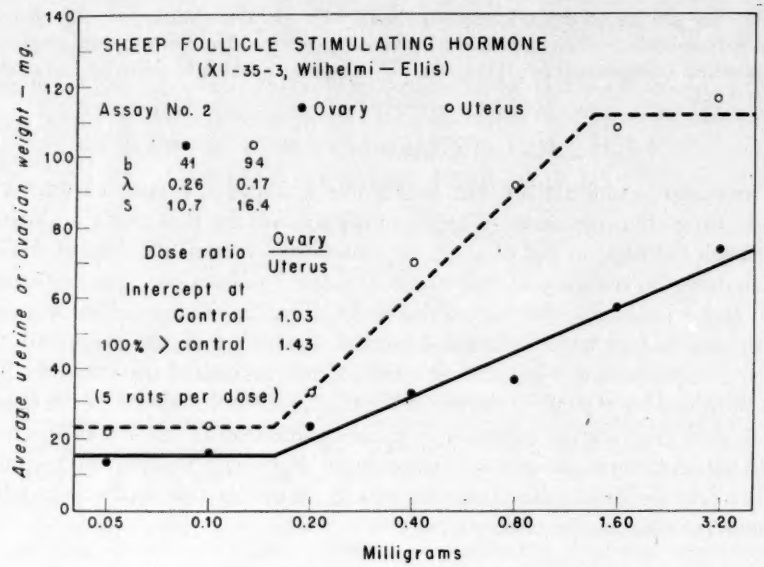

FIG. 2. Seven-point assay of FSH.

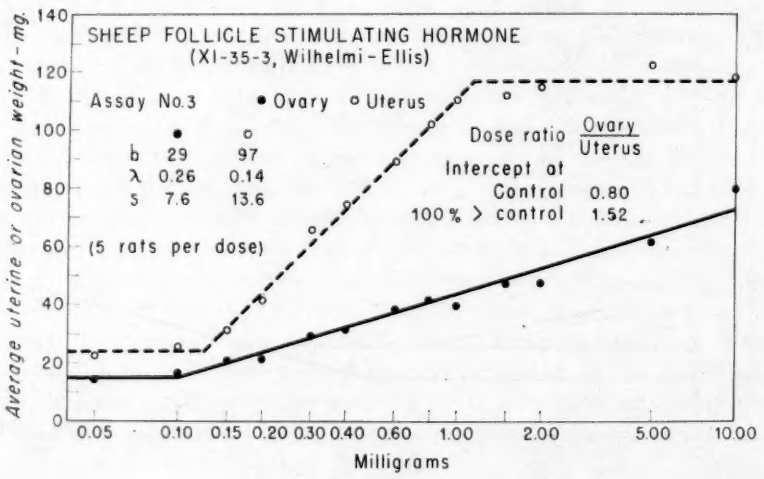

FIG. 3. Thirteen-point assay of FSH.

COMMENT

The parameters of the 3 assays are given in Table 1. Despite the small number of assays, due to the scarcity of FSH, the agreement among the 3 assays is good. Table 2 gives the value in milligrams of FSH for the intercepts of the ovarian and uterine curves at their respective control weights (approximately 15 mg. for the ovary and 25 mg. for the uterus) and at responses equivalent to a doubling of the control weights. These values were averaged and divided by the mean value for the intercept of the ovarian line at its control level (0.123 mg.). This intercept is pivotal, for it allows the construction of a composite curve that can be compared directly with similarly constructed composite curves for other gonadotropins (1, 2).

This intercept was assigned a value of x and plotted as 1 on semilog paper (Fig. 4). The remaining values then were plotted as multiples or fractions of x (given in Table 2). The control lines (approximately 15 mg. for the ovary and 25 mg. for the uterus) then were connected to x and 1.24x,

TABLE 1. PARAMETERS OF THE ASSAY OF FSH

Exp. No.	Ovary		Uterus		Dose ratios—intercept at:	
	b	λ	b	λ	Control	100% >control
1	25	0.19	110	0.12	0.50	1.27
2	41	0.26	94	0.17	1.03	1.43
3	29	0.26	97	0.14	0.80	1.52
Mean	31	0.24	100	0.14	0.78	1.41
SeM	4.8	0.02	4.9	0.01	0.15	0.18

b = Slope.
λ = Index of precision.
SeM = Standard error of mean.

TABLE 2. INTERCEPTS OF DOSE-RESPONSE LINES OF OVARY AND UTERUS (MILLIGRAMS OF FSH)

Exp. No.	Ovarian at control	Uterine at control	Ovarian at 100% >control	Uterine at 100% >control
1	0.09	0.180	0.370	0.29
2	0.16	0.155	0.400	0.28
3	0.10	0.124	0.335	0.22
Mean	0.123	0.153	0.368	0.263
÷0.123	x	1.24x	2.99x	2.13x

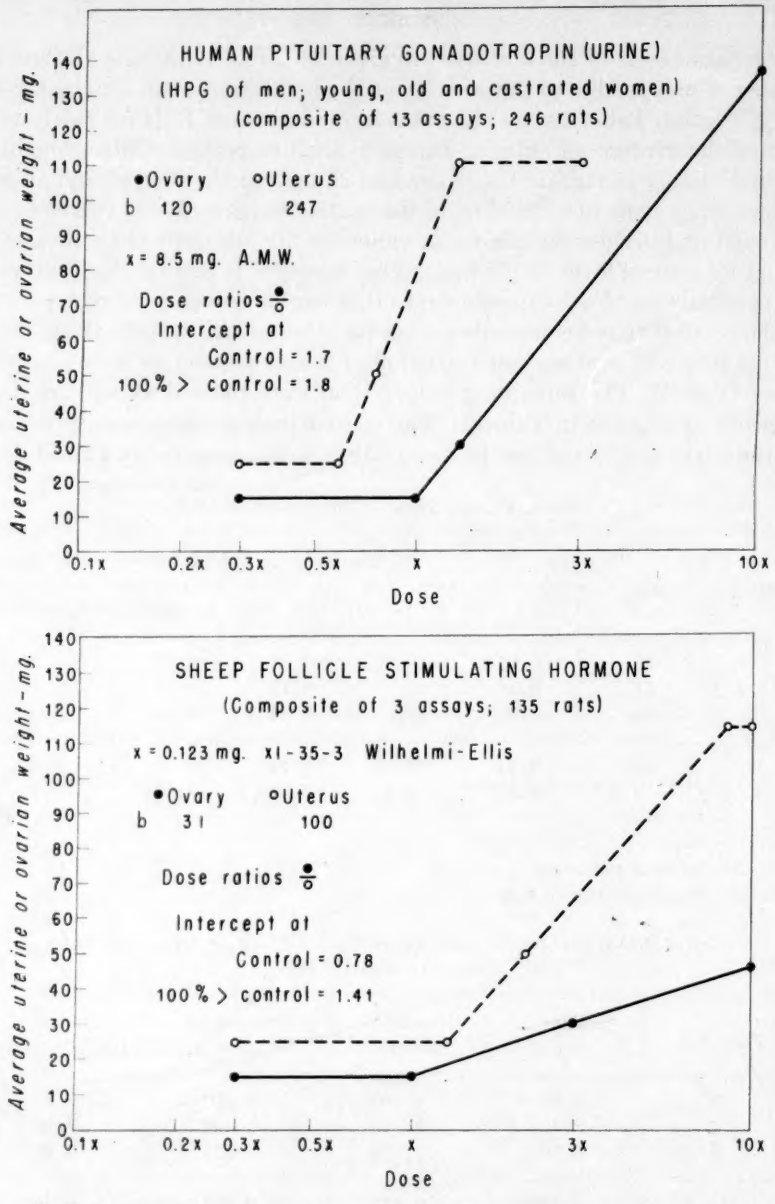
HUMAN PITUITARY GONADOTROPIN (URINE)
(HPG of men; young, old and castrated women)
(composite of 13 assays; 246 rats)
•Ovary ○Uterus
b 120 247
x = 8.5 mg. A.M.W.
Dose ratios •/○
Intercept at
Control = 1.7
100% > control = 1.8
Average uterine or ovarian weight - mg.
Dose
0.1x 0.2x 0.3x 0.5x x 3x 10x
SHEEP FOLLICLE STIMULATING HORMONE
(Composite of 3 assays; 135 rats)
x = 0.123 mg. xi-35-3 Wilhelmi-Ellis
•Ovary ○Uterus
b 31 100
Dose ratios •/○
Intercept at
Control = 0.78
100% > control = 1.41
Average uterine or ovarian weight - mg.
Dose
0.1x 0.2x 0.3x 0.5x x 3x 10x

FIG. 4. Composite assay of HPG and FSH plotted on exactly the same co-ordinates.

respectively. These lines then were extended from these points to the values of 2.99x and 2.13x, respectively, for the ovarian and uterine lines. The curves were extended through these points. The slopes of the lines thus obtained must and actually do have mean values of 31 and 100 for the ovary and uterus, respectively. No data were available for the asymptotic level of the ovarian curve because of insufficient material; hence, the ovarian curve is incomplete. For the uterus, however, the asymptotic value was determined as the mean of the 2 asymptotic values in Figures 2 and 3, each of which is the mean value of several points. This mean asymptotic response was 114 mg. Consequently, the uterine line was extended until it intercepted the asymptotic level of 114 mg.

Comparison of the parameters for the assay of FSH and HPG is given in Table 3 and Figure 4. The slope of the ovarian curve of HPG is more

TABLE 3. PARAMETERS OF THE ASSAY OF HPG AND FSH (SHEEP)

Gonadotropin	Slopes		Dose ratios—intercept at:		Type of curve		Asymptotic response, mg.	
	Ovary	Uterus	Control	100% > control	Ovary	Uterus	Ovary	Uterus
HPG	120	247	1.7	1.8	Linear, log dose	Linear, log dose	300	112
FSH	31	100	0.8	1.4	Linear, log dose	Hyperbolic	?*	114

* Not known because doses greater than 10 mg. of FSH were not available.

than three times greater than that of FSH. The slope of the uterine curve of HPG is two and a half times greater than that of FSH. The dose ratios for the intercept at the control weights are also different. The dose ratio for the zero, or control, intercept is twice as great for HPG as it is for FSH. In effect, this means that uterine stimulation occurs at 0.6 of the dose of HPG that is required to induce ovarian growth. For FSH, uterine stimulation does not take place until after ovarian stimulation has occurred, the threshold dose being 1.2 greater. Thus, on this basis, HPG is twice (1.2 ÷ 0.6) as effective as FSH in inducing the secretion of estrogen. Since the dose ratio at a weight that is 100 per cent greater than the control weight depends also on the slopes of the 2 curves, the same ratio may be obtained when both uterine and ovarian slopes for one gonadotropin are equally different from the respective slopes for another gonadotropin. This accounts for the dose ratios of 1.8 for HPG and 1.4 for FSH at double the control weight. The approximate similarity in these values is due to the

fact, already noted, that the uterine slope for HPG is two and a half times that of FSH, and the ovarian slope is three times greater than that of FSH; hence, both values for HPG are approximately equally different from those of FSH, respectively. The ovarian response to both gonadotropins is a linear function of the log of the dose. The uterine response for HPG is also a linear function of the log of the dose, but that of FSH is not. Comparison of the composite curves for HPG and FSH plotted on exactly the same co-ordinates shows that these 2 materials are entirely different gonadotropins (Fig. 4). It should be apparent that the widespread use in the clinical literature of the term "urinary FSH" for HPG is incorrect.

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THE QUANTITATIVE DETERMINATION OF PROGESTERONE AND PREGNANEDIOL IN HUMAN PLASMA

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ABSTRACT

A chemical method is described for the quantitative determination of progesterone and pregnanediol in human plasma. The sensitivity of the method is demonstrated by its ability to detect as little as 1.0 μg. of progesterone or pregnanediol in a 10-ml. sample of plasma. Its accuracy was tested in recovery experiments in which progesterone was added to male plasma before, and pregnanediol was added before and after, enzymic hydrolysis. In these experiments, recoveries of the order of 85–95 per cent were obtained with amounts of the steroids ranging from 0.2 μg. to 0.9 μg. per ml. of plasma. Evidence for the specificity of the method is presented. In the case of progesterone, this is supported by the results of a paper chromatographic study by the method of Zander and Simmer. In the case of pregnanediol, it was found that the steroid determined by the method has an Rf value identical with that of pure 5β-pregnane-3α: 20α-diol and a closely similar absorption spectrum in concentrated sulphuric acid. A preliminary account is given of the clinical application of the method.

INTRODUCTION

DURING the past five years, work has been carried out in this laboratory upon the development of a chemical method for the quantitative determination of progesterone and pregnanediol in human plasma. The progress made upon this project has been the subject of 2 preliminary reports (1, 2). The present report describes in detail the combined procedure for the determination of both steroids, together with the evidence for the identity of the final products. As indicated previously (1), the task has been made more difficult by the fact that the main objective has been the development of a method of sufficient sensitivity to permit the quantitative determination of the endogenous steroids in single (5.0 to 10.0 ml.) samples of peripheral venous blood.

In 1953 and 1954, Edgar (3) and Zander and Simmer (4), working in-

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dependently, developed procedures for the determination of progesterone based upon the extraction technique of Butt *et al.* (5), followed by paper chromatography (6), elution and ultraviolet spectrophotometry (7). Edgar (8) applied his technique to studies upon the ovarian vein blood of domestic animals. Zander (9, 10), in studies upon the peripheral venous blood in human pregnancy, used infrared spectroscopy and other methods to determine the identity of the suspected progesterone and accomplished the isolation of the hormone from this source. Zander and his colleagues (11) have since obtained valuable information upon the concentration of progesterone in the placenta, other tissues and fluids. However, the method of Zander is not applicable to the analysis of a series of single samples of convenient volume, and the values reported for pregnancy plasma refer to the application of the technique to pooled extracts (10).

Pregnanediol has not hitherto been isolated from human plasma, but evidence for its presence following intravenous administration of progesterone has been reported by one of us (I.F.S.) (12a, b). The procedure used in this earlier work involved precipitation of microgram amounts of pregnanediol from ethanolic solution with water and incorporated a chromatographic step, previously introduced for the determination of relatively large amounts of progesterone (13). The determination was semiquantitative and the method was insufficiently sensitive to determine the small amounts of metabolite of endogenous origin. However, it afforded a starting point for the present investigation.

The subsequent program has involved extensive investigation of different methods of extraction, purification, chromatography and spectrophotometry. Throughout these studies, the main objective has been to achieve a relatively simple technique, and so to facilitate serial analyses and minimal losses. Perhaps the most important step in extraction has proved to be pre-treatment of the diluted plasma with sodium hydroxide and the use of relatively large volumes of the solvent mixture (15:1). Since our preliminary communication (1) it has been reported that alkali pretreatment also proves useful in the extraction of large volumes of plasma in a study in domestic animals (14). The extract obtained by the present technique is clear and colorless and, although centrifugation is used routinely, there is no emulsion formation. For the determination of plasma pregnanediol, extraction is preceded by enzymic hydrolysis with β-glucuronidase. This must be carried out upon a separate sample of plasma because it is technically difficult to repeat the method upon pre-extracted plasma. The extract obtained is also clear and colorless, with an even more rapid separation into the 2 phases following the rapid stirring employed in the extraction. This was not the case when undiluted plasma was extracted in the same way. The 2 other steps requiring careful investigation were the

chromatography of this extract upon a column of standardized alumina and the choice of spectrophotometric end-points. In the case of progesterone, preliminary investigations involved the formation of the dinitrophenylhydrazone and the thiosemicarbazone. Formation of the thiosemicarbazone has been used by other workers (15, 16), and more recently dinitrophenylhydrazone formation has been employed prior to alumina chromatography (17). The thiosemicarbazone proved useful for our purpose and has the advantage of higher sensitivity than occurs with ultraviolet absorption at 240 mμ. Formation of progesterone dithiosemicarbazone was employed in the present study for a period of two years, during which time the concentration of progesterone was calculated from a calibration curve based upon the Allen (18) correction factor obtained with the pure steroid. Thiosemicarbazone formation was abandoned, however, due to its liability to interference by trace impurities in the solvents or extracts, and attention was turned to the formation of the iso-nicotinic acid hydrazone. This hydrazone, which has a maximal absorption at 380 mμ, has been used for the estimation of relatively large amounts of Δ^4-3-ketones in oily solution (19) and more recently for the estimation of Δ^4-3-keto-corticosteroids in human plasma (20). It was adapted to a micro scale, using 0.6 ml. of reagent and reading in a micro cuvette. As in the case of the thiosemicarbazone, the calculation was based on the Allen correction factor, and daily use of this procedure over a further period of fifteen months showed that it is less liable to interference than the former method. A calibration curve of the Allen factors obtained with pure progesterone is unaffected by the addition of eluates from the chromatography of male plasma prior to development with the reagent.

Choice of eluting solvents and the sub-division of the chromatographic fractions yields a clear-cut separation between the fraction containing pregnanediol and the fraction or fractions containing nonspecific chromogenic contaminants. Due to this, it has proved possible to employ the sulphuric-acid reaction on a micro scale for the determination of plasma pregnanediol, again making use of the Allen correction factor. In our experience (involving several hundred chromatograms) this method of determination of the pregnanediol fraction is less liable to interference than is the iso-nicotinic acid reaction.

The conditions finally adopted were the result of a large number of recovery experiments in which 1.0 to 10.0 μg. of progesterone and pregnane-3α:20α-diol were added to 10 ml. of plasma from male subjects. These recovery experiments were controlled by repeated calibration curves, pure solution recoveries from water, and reagent and plasma "blank" controls. A recovery experiment to test only the chromatographic step was carried out at weekly intervals.

MATERIALS

1. *Solvents and reagents*

(a) *Ether.* Anesthetic ether B.P. (May & Baker Ltd., England), washed once with 1/10 volume of saturated aqueous solution of ferrous sulphate and twice with distilled water; then distilled slowly on a steam bath immediately before use.
(b) *Methylene chloride* (British Drug Houses Ltd.), redistilled through a Vigreux fractionating column (length 1 meter) immediately before use.
(c) *n-Hexane,* low in aromatic hydrocarbons (Hopkin & Williams Ltd.) and *Chloroform,* Analar grade (Hopkin & Williams Ltd.), redistilled through a 1-meter Vigreux fractionating column immediately before use.
(d) *Sulphuric acid,* concentrated, nitrogen free, micro-analytical reagent (J. W. Towers Ltd., England).
(e) *Iso-nicotinic acid hydrazide* (L. Light & Co. Ltd., England).
(f) *β-glucuronidase.* Beef preparation, 5,000 units per ml. ("Ketodase"—Warner-Chilcott Laboratories, New York or Wm. R. Warner & Co. Ltd., England).
(g) *Heparin* B. P., pure, (technical grade not satisfactory for this purpose), 96.5 units per mg. (British Drug Houses Ltd.).
(h) *Ethanol,* pure, absolute ethanol, Analar grade "R.R." (Hopkin & Williams Ltd.).
(i) *Alumina,* neutral, activity Grade I (purchased from M. Woelm, Ltd., Germany via L. Light & Co. Ltd., England).

Experience over the past four years has shown that there is minimal variation in the activity of this alumina from batch to batch. It is deactivated by adding amounts of water varying from 4.0 to 4.8 ml. per 100 Gm. This is carried out in a round-bottomed flask which is shaken mechanically at maximum speed for one hour. A water content of 4.8 per cent is used for the separation of the free steroids, but a content of 4.0 per cent gives more clear-cut separation of the pregnanediol from other sulphuric-acid chromogens and is used for the chromatography of the extract after hydrolysis.

2. *Glassware and other materials*

(a) Round-bottomed flasks, condensers (double surface) and distillation equipment—all with ground-glass joints.
(b) Centrifuge bottles, 250-ml., with ground-glass necks to fit all-glass siphon.
(c) Chromatographic columns: straight, diameter exactly 10 mm., length approximately 20 cm., with bulb reservoir of volume approximately 50 ml. and precision-bore oblique stop-cock.
(d) Tubes for chromatographic fractions: 15-ml. capacity, with ground-glass stoppers. (For the sulphuric-acid reaction the tubes and stoppers are fitted with glass lugs to hold a pair of 4-cm. springs.)
(e) Micro cuvettes: fused glass; capacity 0.8 ml. with light pathway 10 mm. (Unicam Instruments Ltd., Cambridge. England).
(f) Filter paper: fluted papers (Green's 9 cm. diameter), discs (10 mm., Whatman No. 1.), and cotton wool used in chromatographic column are all cleaned by treatment for forty-eight hours in a Soxhlet apparatus with pure absolute ethanol and dried in oven.

Cleaning. With the exception of the micro cuvettes, all glassware is soaked in chromic-acid solution after use, than in dilute acetic acid (½ per cent) and rinsed thoroughly with water and then distilled water. In addition, the chromatographic

columns and tubes are washed with hot ethanol and distilled water and dried before use.

(g) Ultraviolet and visible spectrophotometer, S.P. 500 (Unicam Instruments, Ltd., Cambridge, England). This has been modified in the laboratory for use with the micro cuvettes. A micro adjustment has recently been made available by the same firm.

METHODS

A. *For Progesterone and Pregnanediol*

Procedure

In general, 10 ml. of plasma is used for the analysis. Smaller volumes of plasma may be used when the concentration of progesterone or pregnanediol is high (*e.g.*, raised by administration of the hormone), and larger volumes (up to 20 ml.) when the concentration is exceptionally low.

Approximately 20 ml. of blood (drawn by a non-greased syringe) is collected into a centrifuge tube containing 0.2 ml. (200 units) of heparin, B.P. This is centrifuged for twenty minutes at 2,000 r.p.m. The plasma layer is carefully removed. Its volume is measured and it is transferred to a centrifuge bottle.

An equal volume of $\frac{1}{3}N$ sodium hydroxide is added and the bottle is shaken mechanically for five minutes. A 10-ml. portion of the diluted alkaline plasma is transferred to a second centrifuge bottle. The pipette used for this transfer is carefully washed with the solvent mixture. It is important to note that the extracts from these twin centrifuge bottles are subsequently pooled.

The solvent mixture consists of 20 per cent methylene chloride and 80 per cent ether by volume. To each centrifuge bottle is added 150 ml. of solvent mixture and extraction is effected by stirring rapidly for two minutes with a mechanical stirrer. The spiral glass rod of the stirrer is carefully washed down after use. The centrifuge bottles are then spun for ten minutes at 2,000 r.p.m. (during centrifugation the tops of the bottles are covered by cellophane caps). After centrifugation, the extract (upper phase) is siphoned through fluted filter papers into 500-ml. round-bottomed flasks (long-necked). Care is taken not to transfer any plasma, and the extract from each pair of centrifuge bottles is transferred into the same flask. The extract is then evaporated to a volume of approximately 20 ml. by distillation on a steam bath. It is convenient to use a battery of condensers for this purpose and simultaneously to proceed with the second extraction.

The diluted plasma is extracted three times, each with 150 ml. of solvent mixture. The residue from the third distillation is evaporated completely to dryness under negative pressure on a steam bath and stored in the dark in readiness for chromatography on the following day.

The dried extract is dissolved in 20 ml. of freshly redistilled n-hexane, warmed thoroughly on a steam bath and allowed to stand for two hours to ensure complete solution of the steroids. The columns are prepared by allowing the alumina to settle after being poured into the stem which has been filled with n-hexane. The column of alumina (6 cm. in length and 1 cm. in diameter) is supported by a small plug of washed cotton wool, and a disc (1 cm.) of washed filter paper is applied to the top of the column and tapped into place with a glass rod. The level of n-hexane is allowed to fall to the top of the column of alumina. Chromatography is carried out as follows:

1. *n-Hexane:* after transfer of the extract to the column, the extraction flask is washed three times with 10 ml. of n-hexane and these washings are added together to the column. The eluate is discarded.
2. *5% Chloroform in n-hexane:* 15 ml. of this solution is added to the column and the eluate discarded.
3. *20% Chloroform in n-hexane:* a 10-ml. portion is added separately six times. The first 10-ml. eluate is discarded and the remainder is collected into 5 separate stoppered test tubes (capacity, 15 ml.) which have been numbered.
4. *Chloroform:* 5 ml. of chloroform is added twice and 10 ml. twice, yielding 4 eluates, which are collected into the special stoppered tubes used for the sulphuric-acid reaction.

The 5 tubes from 20% chloroform in n-hexane and the 4 tubes containing the chloroform eluates are evaporated to dryness under a nitrogen manifold on a steam bath and stored in a desiccator until thoroughly dry. It is convenient to run the color reactions and standards on the third day.

Color reactions

1. *Reaction with iso-nicotinic acid hydrazide.* The reagent is prepared as follows: 50 mg. of the hydrazide is weighed into a 100-ml. volumetric flask. Approximately 60 ml. of ethanol (the purest grade of absolute alcohol) is added and the reagent is dissolved by gentle heating. After cooling slowly to room temperature, 0.06 ml. of concentrated hydrochloric acid is added; the solution is again allowed to cool, and is finally made up to the mark with ethanol. The reagent is tested by formation of the hydrazone with pure progesterone before use. The molecular extinction coefficient is of the order of 11,800 — in agreement with the coefficient obtained when the reaction was used on a larger scale (19).

This reaction is applied to the 5 eluates of 20% chloroform in n-hexane. To each tube is added 0.6 ml. of the reagent. The tubes are stoppered tightly and maintained at 25° C. in a thermostatic bath for one hour. The presence of hydrazone is revealed by the formation of a pale yellow color. The solutions are transferred to the micro cuvette and are read against the reagent. Routinely, readings are taken at 3 wave lengths: 360 mμ, 380 mμ and 400 mμ and a factor derived as suggested by Allen

(18). The pure steroid yields an absorption maximum at 380 mμ. The concentration of progesterone is calculated by reference to a calibration curve consisting of a series of Allen factors derived from amounts of pure progesterone ranging from 1μg. to 10μg. Depending upon the exact activity of the alumina, the progesterone appears in the third and fourth eluates of 20% chloroform in n-hexane.

2. *Sulphuric-acid reaction.* This reaction is applied to the 4 eluates from pure chloroform. To each tube is added 0.6 ml. of pure concentrated sulphuric acid, great care being taken to prevent contamination of the reagent before and during the reaction. The tubes are tightly stoppered and the small springs are attached. The tubes are then plunged into a boiling water bath for exactly five minutes and then cooled immediately in a beaker containing crushed ice. The solution is transferred (by drawn out Pasteur pipette) to the micro cuvette and read on the spectrophotometer against similarly treated sulphuric acid. The sulphuric-acid chromogen yielded by the pure steroid is yellow in color and has an absorption maximum at 420 mμ. Readings are taken at 400 mμ, 420 mμ and 440 mμ. The Allen factor is calculated and the concentration derived from amounts of pure pregnane-3α: 20α-diol ranging from 2 μg. to 10μg. Although readings are made routinely at 3 wavelengths, complete spectra are read as required.

B. *For Conjugated Pregnanediol*

Hydrolytic procedure

The plasma (in a centrifuge bottle) is adjusted to a pH of approximately 4.5 by the addition of 1*N* hydrochloric acid (spot-testing on indicator papers which register color change to the nearest 0.3 unit). To this is added 0.25 ml. of sodium acetate buffer of pH 4.5 (4*M*), 20,000 units of penicillin (0.2 ml. of aqueous solution) and 10,000 units of β-glucuronidase (2 ml. of Ketodase solution). The bottle is stoppered and incubated at 37° C. for a minimum period of twenty-four hours.

At the end of the incubation period, the alkali (10 ml. of $\frac{1}{3}N$ NaOH) is added, the bottle is shaken and the alkaline plasma is divided as before for extraction. The remainder of the procedure is exactly as described previously, with the exception that the eluates from 20% chloroform in n-hexane are discarded.

Time Required

One assistant can conveniently extract four 10-ml. samples of plasma in four hours (preceded by distillation of ether and methylene chloride). Distillation of n-hexane and chloroform and the chromatography occupy the major part of the following day, and the carrying out of the color reactions and the spectrophotometry occupy a further three hours.

RECOVERY EXPERIMENTS

The results obtained in the following types of recovery experiment are shown in Tables 1 and 2.

TABLE 1. PROGESTERONE

Progesterone added (μg.)	Progesterone recovered (μg.)	% Recovery
(a) *Column recoveries*		
9.20	6.65	72
9.20	8.74	95
9.20	8.74	95
9.20	8.37	91
9.20	8.92	97
9.20	9.20	100
5.50	5.25	96
5.50	5.10	93
5.50	4.40	80
5.50	4.90	90
4.60	4.53	97
4.60	4.53	97
4.60	4.60	100
4.60	4.46	97
4.60	4.28	93
4.60	4.60	100
4.55	4.45	97
4.55	4.52	98
4.55	4.52	98
(b) *Recovery of progesterone from water*		
4.44	4.42	98
4.44	3.53	80
(c) *Recovery from male plasma*		
9.20	8.89	97
9.20	8.59	83
9.20	8.89	97
9.20	6.90	75
5.50	4.85	88
5.50	4.80	86
5.50	4.40	80
4.55	3.69	82
4.55	4.20	91
4.55	4.32	95
3.30	3.10	94
2.20	1.80	82
1.10	1.00	91
1.10	0.80	72

TABLE 2. PREGNANE-3α:20α-DIOL

Pregnanediol added (μg.)	Pregnanediol recovered (μg.)	% Recovery
(a) *Column recoveries*		
5.98	5.54	90
5.40	5.24	97
4.55	4.51	97
4.55	4.49	90
4.55	4.53	97
4.55	4.53	97
4.55	4.17	92
(b) *Recovery from male plasma*		
(i) *Non-hydrolyzed*		
4.60	4.42	96
4.60	4.40	94
4.55	4.53	97
4.55	4.49	90
4.55	4.43	95
2.36	1.92	82
(ii) *Hydrolyzed*		
9.72	8.85	91
9.72	9.33	95
9.72	7.78	80
5.98	5.90	97
5.40	5.18	96
5.40	5.24	97
5.40	5.24	97
4.50	4.45	98
2.99	2.80	95
2.99	2.80	95
2.82	1.94	69
2.82	2.64	94
2.82	2.64	94
2.82	2.64	94
2.82	2.64	94
1.99	1.78	92
1.99	1.78	92
1.99	1.62	84

(a) *Column recovery*

As stated in the introduction, the efficiency of the column chromatography is tested repeatedly by recovery experiments in which pure progesterone and pregnane-3α:20α-diol are evaporated to dryness, dissolved in n-hexane and chromatography performed. The recoveries fall within the range 90–98 per cent.

(b) *Reagent and male plasma "blank"*

A further control is provided by running through the entire procedure, substituting water for plasma. This tests all the reagents and excludes unexpected contamination from such sources as the heparin and sodium hydroxide solutions. When all reagents are pure, the spectrophotometric readings yield an Allen factor (D corr.) of ± 0.002.

Recovery experiments have been carried out with male plasma both with and without enzymic hydrolysis. In the fractions for male plasma which correspond to those in which progesterone would appear if present, the Allen factors (D corr.) fall within the range ± 0.005, and in the fractions in which pregnanediol would appear, within the range ± 0.002. The spectrophotometric readings closely resemble those obtained in the reagent "blank."

Satisfactory "blank" readings were a constant finding in the case of the pregnanediol fractions. Difficulty may arise, however, in obtaining satisfactory linearity with the iso-nicotinic acid hydrazone. From time to time, interference may result in negative Allen factors and this can only be eliminated by careful step-wise re-investigation of all possible sources of contamination. It is apparent that the method does not detect progesterone or pregnanediol of endogenous origin in male plasma, but the plasma of nonpregnant women has not yet been investigated.

(c) *Recovery of steroids added to male plasma*

Pure progesterone and pregnane-3α:20α-diol in minimal volumes of ethanol (0.2 ml.) are added to the plasma in amounts ranging from 1.1μg. to 9.2 μg. The plasma is shaken mechanically for five minutes at a high speed and the procedure carried out. In the case of pregnanediol the steroid is added before enzymic hydrolysis. The results are shown in Tables 1 and 2 and the recoveries fall within the range 80 to 95 per cent. Comparison of these recoveries with the results obtained in the column recoveries indicates that the extraction loss is negligible in the case of pregnanediol and is not greater than 5 per cent in the case of progesterone. Although the recoveries prior to hydrolysis indicate that there is again negligible loss during this step, recoveries from pure sodium pregnanediol glucuronidate have not yet been tested.

IDENTITY OF THE FINAL PRODUCTS

Progesterone

The specificity of the method depends mainly upon the chromatographic purification which precedes the final color reaction, since other steroids (*e.g.*, testosterone) yield hydrazones with spectra similar to that of progesterone. Evidence suggesting that the hydrazone determined by the method is in fact progesterone, is as follows:

(i) The linearity of the male plasma "blank," indicating that other hydrazone-forming compounds are not detectable in the appropriate fractions.
(ii) The hydrazone formed by the unknown compound appears in the same fraction as pure progesterone.
(iii) Large amounts of the hydrazone appear in extracts following the intravenous administration of progesterone to human subjects following a control period during which the hormone is not detectable.
(iv) The hydrazone is found in pregnancy plasma and indicates amounts of progesterone which are comparable with those obtained from pooled extracts by the method of Zander and Simmer (4).
(v) The results of paper chromatography.

More direct evidence was made possible by the co-operation of Dr. J. Zander and his colleagues at the Universitäts-Frauenklinik, Köln, Germany, who applied the paper chromatographic and elution steps of the method of Zander and Simmer to the final product of the new method. Dr. Zander reported that the appropriate fractions from the alumina chromatograms contain a substance in a highly pure state with the same Rf value as progesterone. On elution from the paper and spectrophotometry in the ultraviolet, the absorption curve had a clear maximum at 240 mμ (Fig. 1). In order to apply the method of Zander and Simmer (4) to the fractions obtained from single (10 ml.) samples of plasma it was necessary to study plasma obtained following intravenous administration of the hormone. Progesterone was not detected by Dr. Zander in the fractions from the pre-injection control plasma.

Pregnanediol

As in the case of progesterone, the specificity of the method depends upon the purification which precedes the formation of the sulphuric-acid chromogen. Evidence suggesting that the chromogen is one of the pregnanediols is as follows:

(i) The linearity of the male plasma "blank." As mentioned in the introduction, this is a constant finding.
(ii) The sulphuric-acid chromogen appears in the same fraction as pure pregnane-3α: 20α-diol.
(iii) Following intravenous administration of progesterone, the rapid disappearance of the hormone from the plasma is accompanied by a gradual rise in the level of the suspected pregnanediol.

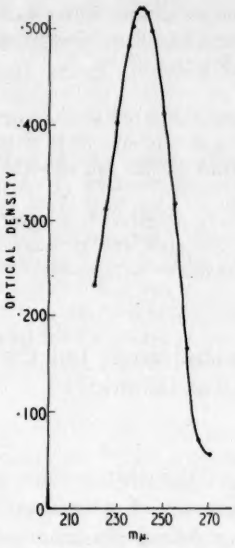

FIG. 1. Ultraviolet absorption spectrum (in methanol) after elution from paper of material with same Rf value as pure progesterone.

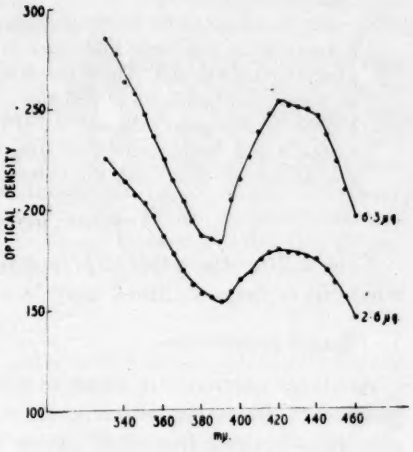

FIG. 2. Sulphuric-acid absorption spectrum after elution from paper of material with same Rf value as pure pregnane-3α:20α-diol.

(iv) The sulphuric-acid chromogen is detected in pregnancy plasma following enzymic hydrolysis.

(v) The results of paper chromatography:

The chloroform eluates from the alumina chromatogram were subjected to chromatography on paper, using a Bush system (6) consisting of iso-octane (25 per cent) toluene (25 per cent) and aqueous methanol containing 15 per cent water (50 per cent). The paper (Whatman No. 1) was washed by running in a tank with 50 per cent aqueous methanol for at least twenty-four hours and dried in an oven. Care was taken to protect the cleaned paper from contamination. The extracts and standard pregnane-3α:20α-diol were applied to the paper, which was inserted into a tank which had been previously equilibrated with both phases. The tank was sealed and, after a further period of equilibration of at least three hours at 32–33° C., the mobile phase (toluene-iso-octane mixture) was added. With the front under observation, the chromatogram was allowed to run for two to two and a half hours, and was subsequently dried in an oven. Strips of the paper were then developed with 10 per cent ethanolic phosphomolybdic acid (21)

In experiments with pure solutions this reagent was found to yield a detectable spot with as little as 3 μg. of pregnane-3α:20α-diol. This procedure was applied to fractions from male plasma, from male plasma to which pregnane-3α:20α-diol had been added, from pregnancy plasma (hydrolyzed) and from plasma obtained after intravenous administration of progesterone. With the exception of male plasma, in all of these fractions a compound was detected in a highly pure state and with an Rf value which was identical with that of pure pregnane-3α:20α-diol. Furthermore, when undeveloped spots were eluted with pure methanol (using

the elution device described by Zander and Simmer) and the eluates were rechromatographed on alumina columns, a sulphuric-acid spectrum closely similar to that of pure pregnane-3α:20α-diol was obtained (Fig. 2).

(vi) Sulphuric-acid spectra:

Fractions obtained by the standard method and subjected to the micro-modification of the sulphuric-acid reaction yield spectra closely similar to that of pure pregnane-3α:20α-diol. There are absorption maxima at 325 mμ and 420 mμ and an absorption minimum at 390 mμ.

(vii) Further work upon the specificity of the method is in progress. In recovery experiments it has been found that pregnane-3α,17α,20α-triol and pregnan-3α-ol-20-one do not interfere with the determination of pregnane-3α:20α-diol.

APPLICATION OF THE METHOD

This will be the subject of a separate communication, but the results which have been obtained may be summarized as follows:

1. *Plasma progesterone*

Analyses carried out upon samples taken at the thirty-sixth week of pregnancy have consistently shown the presence of amounts of progesterone ranging from 0.07 μg. to 0.13 μg. per ml. of plasma. In plasma from placental venous blood the level was of the order of 0.4 μg. to 0.5 μg. per ml. In general, 10 ml. of plasma was used for each analysis, but in certain cases a higher concentration was achieved by applying the method to an extract derived from 20 ml. of pregnancy plasma. These results are in agreement with those obtained for pooled extracts (10, 22) and with an approximation previously suggested for pooled placental blood (23).

Progesterone was administered to human subjects by the intravenous infusion of the hormone in propylene glycol (20 mg. per ml.). Samples of peripheral venous blood were taken from the opposite arm-vein and at frequent intervals over the subsequent six hours. During the infusion, the progesterone level rose rapidly to a sharp maximum and then fell steadily to reach a low level in approximately one hour.

2. *Plasma pregnanediol*

The level of pregnanediol in peripheral venous blood has been studied in 160 samples from 130 women throughout pregnancy. Pregnanediol was detected consistently from the early weeks and rose from levels of the order of 0.05 μg. to 0.10 μg. per ml. of plasma in the first trimester to levels of the order of 0.30 μg. to 0.60 μg. per ml. in the third trimester. During the twenty-four hours following parturition, plasma pregnanediol fell rapidly to an undetectable level.

In metabolic experiments in which the progesterone was infused, as described here, there was a gradual rise in plasma pregnanediol which attained a maximum level approximately one hour after infusion. This was

followed by a slow fall to low levels. These experiments amplify and confirm the earlier work (I.F.S.) (12a) in which progesterone was administered in human serum albumin.

Acknowledgment

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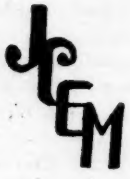

INHIBITION OF THE SODIUM-RETAINING INFLUENCE OF ALDOSTERONE BY PROGESTERONE*†

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ABSTRACT

Progesterone in a dosage of 50 mg. per day elicited a pronounced natriuretic response in a woman with Addison's disease who was treated with aldosterone and cortisone. After the aldosterone had been discontinued, the same amount of progesterone had no influence on urinary sodium excretion.

In acute studies performed in 2 other adrenal-deficient subjects, 150 mg. of progesterone nullified the sodium-retaining effect of a slow intravenous infusion of 20 micrograms of aldosterone. Under the same circumstances, progesterone alone had no influence on urinary sodium excretion. The same quantity of progesterone failed to block the effect of 40 or 50 micrograms of aldosterone.

These results were interpreted as demonstrating that progesterone is an antagonist of aldosterone, presumably at the level of the renal tubules. It was accordingly assumed that the natriuretic influence of progesterone in normal subjects is due to the inhibition of endogenous aldosterone.

In any consideration of the control of sodium metabolism during pregnancy and the luteal phase of the menstrual cycle, this competitive interaction of progesterone and aldosterone must be given a major role.

PROGESTERONE is a natriuretic and chloruretic agent in man (1–3). In subjects with intact adrenocortical function a course of 50 mg. per day intramuscularly induced a prompt rise in urinary sodium excretion which was sustained for from two to six days and produced a net loss of sodium of about 70 to 150 milliequivalents. In each of these subjects urinary sodium excretion dropped below control levels immediately after progesterone was discontinued, and in the ensuing days the amount of sodium and chloride which had been dissipated during the first days of pro-

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gesterone administration was regained. This natriuretic influence was detectable when the dosage of progesterone was as little as 12 to 15 mg. per day. When the dosage was increased to 150–300 mg. daily, the loss of sodium was as much as 396 milliequivalents in seven days.

These effects of progesterone are dependent upon the presence of functioning adrenal cortices. When progesterone was given to adrenal-deficient patients who were receiving constant replacement amounts of cortisone acetate and desoxycorticosterone acetate, the rise in urinary sodium excretion was greater and the duration of the effect was longer than when the same dosage was administered to other subjects. Despite the substantially greater loss of sodium incurred by subjects with treated Addison's disease, there was never any retention of salt after progesterone was discontinued. In 1 case of Addison's disease, progesterone failed to affect sodium excretion when it was given while the patient was treated only with added salt. Very shortly after treatment with cortisone and desoxycorticosterone had been instituted in this patient, a repetition of progesterone therapy induced intense natriuresis and chloruresis. From these results it was concluded that the sodium-dissipating influence of progesterone was probably due to the competitive inhibition at a renal level of the salt-retaining influence of administered desoxycorticosterone and cortisone. It was similarly suggested that the natriuresis induced in normal subjects could be explained by inhibition of aldosterone, the adrenal steroid hormone with the greatest salt-retaining activity (4).

In the studies to be reported here we have endeavored to secure evidence to support the view that the salt-retaining action of aldosterone may be inhibited by progesterone.

PROTOCOLS

M. A. (Fig. 1) was a 46-year-old woman with Addison's disease. She was healthy otherwise and there were no signs of tuberculous or other infection of the adrenal glands. During the study she received 25 mg. of cortisone acetate intramuscularly twice a day. She also required desoxycorticosterone acetate for satisfactory management of the adrenal deficiency.

L. K. was a 32-year-old man with Addison's disease apparently due to idiopathic atrophy of the adrenal cortices. During the study he received 12.5 mg. of cortisone acetate orally three times a day, except as noted on the experimental days. His total salt intake was 280 milliequivalents per day. Serum electrolyte concentrations did not vary significantly throughout the studies.

D. E. was a 49-year-old woman with metastatic breast cancer who had been adrenalectomized nine months previously. She was in good physical condition. During the study she received 25 mg. of cortisone acetate orally twice a day, except as noted on the experimental days. Her total salt intake was 117 milliequivalents per day. Serum electrolyte concentrations did not vary significantly throughout the studies.

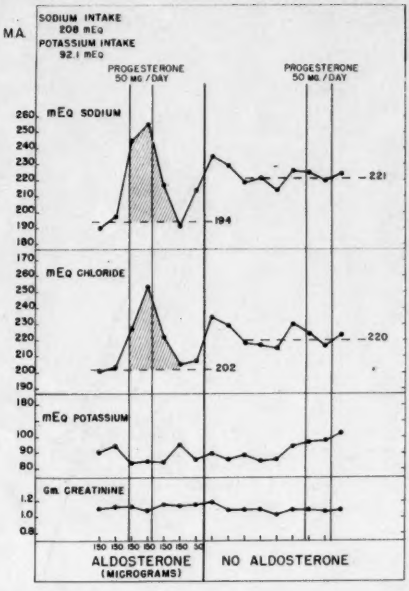

FIG. 1. (*M.A.*, a 46-year-old woman with Addison's disease.) The effects of progesterone on the urinary excretion of sodium, chloride, potassium and creatinine. Throughout these observations she received 50 mg. of cortisone acetate per day intramuscularly. The heavy vertical line separates the period of aldosterone therapy from that without aldosterone. The indicated dosages of aldosterone represent the amounts of biologically active *d*-aldosterone. Broken horizontal lines indicate averages of control values. Oblique hatching indicates significant shifts.

EXPERIMENTAL DESIGN AND METHODS

In the first study (Fig. 1) conventional balance techniques were employed. Patient M.A., a woman with Addison's disease (see protocols), was given a constant adequate diet; 24-hour urine collections were made and checked for completeness by means of creatinine estimations. After reasonable control of the adrenal deficiency had been secured by administration of 50 mg. of cortisone acetate and 1 mg. of desoxycorticosterone acetate per day, the desoxycorticosterone acetate was replaced by 100 micrograms of *d,l*-aldosterone-21-monoacetate in sesame oil given intramuscularly every eight hours (equivalent to approximately 150 micrograms of biologically active *d*-aldosterone (5) each day). Dosages recorded in Figure 1 and subsequently indicate the quantities of the biologically active isomer. After two days of this program, 50 mg. per day of progesterone was given intramuscularly for two days; three days thereafter, aldosterone was discontinued. Seven days later the two-day course of progesterone was repeated.

In the acute studies carried out on the cortisone-treated adrenal-deficient patients, L.K. (Fig. 2) and D.E. (Fig. 3), the effect of a small intravenous dose of aldosterone on electrolyte excretion was determined by administering the hormone under carefully controlled conditions, twelve to eighteen hours after the last dose of oral cortisone. Since the effect of aldosterone would be superimposed on a phase of the diurnal variation in sodium, potassium and chloride excretion prevailing under these conditions, its delineation would require comparison with observations on an identical day in which no aldosterone was given. The influence of progesterone on the effect of aldosterone was determined on a third day by pre-treating the subjects with intramuscular pro-

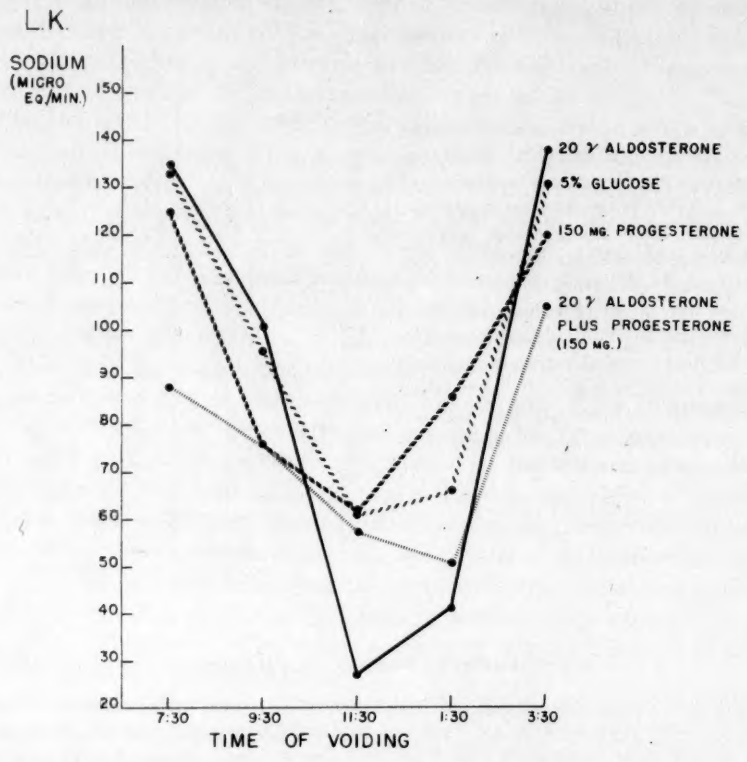

FIG. 2. (*L.K.*, a 32-year-old man with Addison's disease.) The rate of sodium excretion through four experimental days. The values plotted above the times of voiding actually represent the rate of excretion of sodium during the previous two-hour interval, except for the rate at 7:30, which represents the period beginning at 10:30 P.M. the night before. The 20-microgram dosage of aldosterone was the indicated quantity of the biologically active *d*-aldosterone.

gesterone; the effect of progesterone alone was evaluated on a fourth experimental day which was identical with the third except that no aldosterone was given.

Both L.K. and D.E. were given constant maintenance diets and treated with oral cortisone acetate and added salt (see protocols). Twenty-four hour urine collections were made, and evidence of metabolic equilibration was secured before any studies were carried out. Each of the experimental days required breaking the constant regimen; the studies were always separated by two or three days of the constant control program. The day prior to the study, the 24-hour urine collection was broken at 10:30 P.M.; the 10:30 P.M. to 7:30 A.M. collection served as a pre-treatment control urine for the experimental day. At 7:40-7:45 A.M. on the day of the study, an intravenous infusion of 150 ml. of 5 per cent glucose was started and carefully timed to require one hour for its delivery. The patients were supine during this infusion but thereafter were allowed to be ambulant. The morning doses of cortisone acetate and sodium chloride, as well

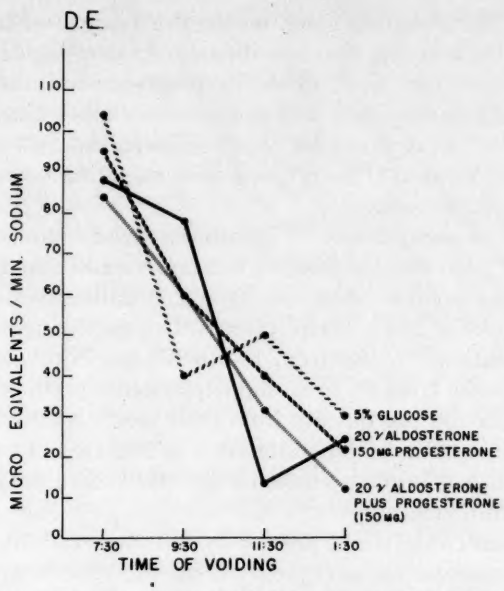

FIG. 3. (*D.E.*, a 49-year-old adrenalectomized woman with breast cancer.) The rate of sodium excretion through four experimental days. For key to charting, see legend of Figure 2.

as breakfast, were omitted. The patients drank 200 ml. of water every hour from 8:00 A.M. through 1:30 P.M. At 1:30 lunch was served (the same for each experimental day). Precisely timed two-hour urine collections were made throughout the period of study. These, as well as the ones from 10:30 P.M. to 7:30 A.M., were analyzed for sodium, chloride and potassium. At 3:30 P.M. the experiment was terminated, and the cortisone acetate and salt which had been withheld were given. The aldosterone, dissolved in ethanol (200 micrograms per ml. of *d,l*-aldosterone-21-monoacetate), was added to the 150 ml. of 5 per cent glucose on the days on which it was to be given. Progesterone dissolved in sesame oil and 20 per cent benzyl-benzoate was given intramuscularly in divided dosage—100 mg. at 5:30 A.M. and 50 mg. at 7:30 A.M.

Urinary chloride and creatinine were determined as noted previously (6). Sodium and potassium measurements were made using the Perkin-Elmer internally standardized flame photometer. Diluted urine was loaded with a gross excess of potassium when measuring sodium, and a similar excess of sodium when potassium was determined.

RESULTS

When progesterone was given to Patient M.A. (Fig. 1) while her adrenal deficiency was being treated with cortisone and aldosterone, the usual effect on sodium and chloride excretion was observed. Urinary sodium rose sharply from the baseline of 194 milliequivalents per day to a peak of 255 milliequivalents and then dropped to the control level on the second post-

treatment day. The elevation noted on the third day after treatment was associated with the lowering of aldosterone dosage to a single injection of 50 micrograms. The sodium loss induced by progesterone totalled 135 milliequivalents. Chloride excretion followed a course which closely paralleled that of sodium but, as usual under these circumstances, the total loss was less (95 milliequivalents). Urinary potassium excretion was probably uninfluenced by progesterone.

Following the discontinuance of aldosterone, the sodium and chloride balances shifted from slightly positive to negative and a new baseline was assumed. Urinary sodium then averaged 220 milliequivalents per day with a daily intake of 208 milliequivalents. During this period the serum sodium concentration dropped from 134 to 123 milliequivalents per liter; and that of chloride, from 93 to 85 milliequivalents per liter. Urinary sodium and chloride did not deviate from their newly established baselines when the administration of progesterone was repeated. The excretion of potassium which had begun to increase slightly before progesterone was given was also uninfluenced by it.

This experiment confirms the previously reported study in which it was shown that the saluretic action of progesterone was observed only while the organism was under the influence of salt-retaining corticoids. Both cortisone and desoxycorticosterone were given in that instance (1), but the latter undoubtedly provided most of the sodium-retaining activity. In Patient M.A. (Fig. 1) the cortisone acetate had virtually no salt-retaining action, and it is clear that progesterone was only salt-dissipating when given while the patient was receiving the powerful sodium-retaining hormone, aldosterone.

The acute experiments in Patients L.K. and D.E. (Figs. 2 and 3) were carried out because in M.A. (Fig. 1) and in the previously reported study (1), when no aldosterone or no desoxycorticosterone and cortisone were given to the subjects, the failure of progesterone to be a sodium-dissipating agent may not have been due primarily to the fact that there was no sodium-retaining corticoid present to be inhibited or antagonized. In both subjects there was a mild to moderate negative sodium balance. The serum concentrations of sodium and of chloride had declined, and the patients' general condition, though reasonably good, was deteriorating. These factors could also be offered as explanation for the failure of progesterone to act as a natriuretic. It was hoped that the single-day studies might provide added evidence that the sodium-retaining influence of aldosterone is indeed antagonized by progesterone. This would be demonstrated if the aldosterone effect were blocked by adding progesterone, and on a subsequent day progesterone alone were shown to have no direct influence on sodium excretion.

Under the conditions of these studies the pattern of sodium excretion

during control days (5 per cent glucose infusion) was very similar in Patients L.K. (Fig. 2) and D.E. (Fig. 3). The rate of sodium excretion was relatively high during the overnight period (7:30 A.M. voiding), and then dropped progressively until 11:30 A.M. in L.K. and until 1:30 P.M. in E.D. The rate increased slightly from 11:30 to 1:30 in L.K., and then sharply during the next two hours. In D.E. there was an abrupt return to the overnight rate of sodium excretion from 1:30 to 3:30 (data not shown). The addition of 20 micrograms of aldosterone to the glucose infusion caused a very significant drop in the rate of sodium output during the 9:30 to 11:30 A.M. period. When aldosterone was given to Patient L.K. (Fig. 2) the rate of sodium excretion was 27 microequivalents per minute as compared with 61 microequivalents per minute on the control day. In Patient D.E. (Fig. 3) the drop at this point was from 49 microequivalents on the control day to only 13 microequivalents per minute. A similar suppression of the rate of sodium excretion by aldosterone was noted on 4 other experimental days in the same subjects and in 2 studies in a third adrenal-deficient patient. The suppression of urinary sodium excretion from two to four hours after the start of the one-hour intravenous infusion of aldosterone would thus appear to be the characteristic response.

When the subjects were pre-treated with intramuscular progesterone (with or without aldosterone) the excretory rate for sodium during the critical 9:30–11:30 A.M. interval compared closely with the rates observed on control days. In Patient L.K. these 3 rates were almost identical. In Patient D.E. the values were not grouped so closely, but the rates of excretion when progesterone was injected and when progesterone plus aldosterone was given were more than twice as great as that observed when aldosterone alone was administered. Larger amounts of aldosterone (the equivalent of 50 micrograms of *d*-aldosterone in L.K. and 40 micrograms in D.E.) lowered the sodium excretion to about the same level as did administration of 20 micrograms, but the same dosage of progesterone did not abolish or significantly lessen these aldosterone responses.

Potassium excretion was not systematically affected by any of the hormonal treatments. Urinary chloride tended to vary in parallel with the rate of sodium excretion but, not too suprisingly, since aldosterone primarily promotes sodium retention, the suppression of chloride from 9:30 to 11:30 A.M. was less than that of sodium, and its nullification by progesterone was not uniform.

These acute studies thus confirm the impression gained from the balance study in Patient M.A. (Fig. 1) that the natriuretic influence of progesterone was due to the peripheral antagonism of aldosterone. When given prior to the infusion of aldosterone, progesterone prevented the anticipated suppression of urinary sodium. Since there was no evidence that progesterone had a primary natriuretic action, abolition of the aldosterone

effect must have been due to a blocking of the influence of aldosterone, presumably in the kidney tubules. The failure of progesterone to be salt-dissipating in the absence of aldosterone in Patient M.A. can accordingly be explained on the basis of absence of sodium-retaining hormone which could be antagonized, rather than on the basis of unresponsiveness due to the systemic effects of loss of sodium and chloride from the body.

DISCUSSION

The capacity of progesterone to inhibit the sodium-retaining influence of aldosterone and desoxycorticosterone is probably dependent upon some close structural similarities of these compounds. When a change as small as the introduction of an hydroxyl group (as in 17-α-hydroxyprogesterone) is made, the ability to inhibit salt-retaining corticoids is lost. Several synthetic progestational compounds which, from the chemical point of view, are less closely related to progesterone also failed to elicit a natriuretic response (7). On the other hand the unusual synthetic compounds, 3-(3-oxo-17-β-hydroxy-4-androsten-17α-yl)propionic acid, γ-lactone and its 19-nor analog, have recently been shown to be sodium-dissipating in rats (8) as well as man (9), apparently by virtue of a similar inhibition of aldosterone. In rats there was a suggestion that the relationship between these blocking agents (including progesterone) and aldosterone followed the law of mass action. In the studies reported here, a 20-microgram dose of *d*-aldosterone was inhibited by 150 mg. of progesterone, whereas 40- to 50-microgram doses were not. Such a blockade of aldosterone by progesterone, as well as the apparent chemical similarities, might suggest that progesterone is a relatively weak salt-retaining steroid itself. As a matter of fact, such an action has been demonstrated in dogs (10) and suggested in rodents, in which progesterone in large amounts supports the life of adrenalectomized animals (11, 12). However, in man, no salt-retaining activity has ever been described.

The antagonism of progesterone to the typical sodium-retentive influence of aldosterone permits the assumption that such a blockade of the effects of endogenous aldosterone accounts for the sodium-dissipating effects of progesterone in normal persons. The more limited losses of salt and the prompt post-treatment recovery of lost sodium in these subjects is probably explained by a compensatory increase in the secretion of aldosterone. A similar adrenal response to the progesterone secreted during the normal menstrual cycle and during pregnancy would be anticipated. The greatly increased excretion of aldosterone noted during normal pregnancy (13) probably reflects its enhanced secretion, in part at least, in response to the large amount of progesterone secreted by the placenta and the corpus luteum of pregnancy.

It has been suggested that in some circumstances the adrenal cortex may secrete a sodium-dissipating agent (14). In the light of the findings reported here, it is conceivable that this presently unidentified secretion may be progesterone.

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RADIOCALCIUM STUDIES OF BONE FORMATION RATE IN HUMAN METABOLIC BONE DISEASE*

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ABSTRACT

Calcium[45] tracer studies were performed in 12 adult human subjects with and without bone disease. The size of the miscible calcium pool averaged slightly less than 100 mg. Ca/Kg. in all subjects, except for 2 patients with osteitis deformans in whom the pools were four times that size. A direct relationship between the size of the pool and the rate of bone formation was demonstrated, and attributed to the variable contribution of the mass of newly formed bone to the miscible calcium pool.

Evidence is cited which indicates that this pool, despite its theoretical complexity, behaves as a single compartment. The turnover of this pool is shown to be greatly in excess of combined excretory loss and is therefore presumed to include loss of tracer into non-exchanging bone, in the process of bone formation. A method is described whereby this bone uptake may be estimated quantitatively.

On the basis of a study in 1 normal adult in the present series, and of available studies in the current literature, the normal rate of bone formation is estimated to be approximately 9 mg. Ca/Kg./Day (mean for 10 subjects: 9.1 ± 3.9 S.D.). The rates of bone formation in 6 patients with osteoporosis uncomplicated by parathyroid disease were clearly within this tentatively normal range (9.4 ± 1.07). A much reduced rate of bone formation was found in 1 patient with uncomplicated hypoparathyroidism, a slightly elevated rate in a young male with familial hyperostosis, and greatly elevated rates in 2 patients with osteitis deformans.

From the time of return of bone isotope into the pool, the biologic life of the shortest-lived species of bone was estimated, and measurement was made of the proportion of total bone formation represented by this recycling. In general, the time required for this earliest recycling seemed to vary inversely with the postulated turnover of bone.

THE adult skeleton, once considered to be altogether inert, has been shown to be subject to continual turnover, just as are other body tissues. This turnover has been presumed to be of two sorts: 1) actual phys-

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ical destruction of structural units with replacement anew by similar units, and 2) physicochemical exchange processes between the solid phase of the bone substance and the soluble phase of the body fluids, with no net local change in structure.

The possible importance of exchange processes to calcium homeostasis and its disorders has been recently discussed in reviews by Neuman and Neuman (1), Howard (2), and McLean (3). Whatever role such exchange may play in bone disease, alterations in the rates of formation and destruction of structural bone elements are also importantly involved. The very definitions of certain bone diseases assume altered bone formation or destruction; osteoporosis and osteitis deformans are cases in point. Heretofore these alterations have been inferred largely from essentially static studies of bone pathology or from classic balance techniques. Direct measurement of the rates involved, however, is not possible with these methods.

In the analysis of steady-state physiologic and pathophysiologic systems, such as presumably exist in bone diseases, radioactive tracers have proved to be of invaluable assistance. Nevertheless, although use of bone-seeking isotopes has produced much information on isolated aspects of bone salt metabolism in animals, comparatively little work has thus far been reported on derangements of bone metabolism in the whole animal. One immediately obvious reason is the difficulty in assigning meaning to tracer data derived from the intact organism.

A fully accurate model system for the interpretation of isotope data, based upon present knowledge of calcium metabolism, would consist of an open system having at least 5 or 6 compartments, with fairly complex internal recycling, and with a series of external, long-period delay cycles. In such a system, using but a single tracer, and having ready access to only 1 of these compartments, it is improbable that an explicit solution could be derived for a single interior rate constant, such as the rate of new bone formation. Such a model system can, however, be made to yield useful data by making certain simplifying assumptions—the validity of the results depending upon the operational accuracy of the simplifications.

At least 3 somewhat simplified models have been proposed thus far in various studies of strontium and calcium behavior in man and animals. Bronner and Harris (4) proposed what amounts to a 4-compartment open system, in which bone itself was treated as one of the equilibrating compartments, and has been cited to this effect by Robertson (5) in his recent review of tracer theory. As will be seen from evidence presented later, bone probably should not be treated as an equilibrating compartment. In any case, such an assumption would introduce serious difficulties into the measurement of rates of bone formation.

Bauer and Ray (6) analyzed their isotope data using an analogue com-

puter. Making certain assumptions, they found their results compatible with a 4-compartment open system in which bone is not treated as an equilibrating compartment but as an internal route of loss. In this paper, as in the earlier publications of Bauer, Carlsson and Lindquist (7–9), calcium involved in bone formation ("accretion") is considered as removed from the "miscible calcium pool" in exactly the same sense as excreted calcium is removed from the pool. In simplifying this formulation, these authors suggested a single-compartment open system encompassing the 4 observed equilibrating compartments, with loss of isotope from this compartment both by excretion and by incorporation into non-exchanging bone. Although the work on which this single-compartment model was based dealt with strontium^{85}, virtually the same model is implied in previous work by Bauer and his co-workers using calcium^{45} and other bone-seeking isotopes.

The present authors, in work commencing before the presentation of the latter formulation by Bauer and his co-workers, had concluded that such an admittedly simplified model system was both workable and accurate. It is the purpose of this communication to describe this model in detail, to examine its assumptions in the light of presently available knowledge of calcium metabolism, and to describe its preliminary application in 12 patients with various metabolic bone diseases.

METHODS

Conceptual model

The model used in the present studies to interpret the isotope data is given in Figure 1, and consists of a single-compartment system with 2 routes of loss (u,s) representing urinary and endogenous fecal excretion, and recycling by way of a series of long-period release mechanisms through a third route (b), representing incorporation of isotope into non-exchanging bone and its subsequent return into the pool at the time of osteolysis. The pool is labeled instantaneously (by intravenous injection), and the route of entry, d, from the diet remains unlabeled. The route of entry, $-b$, representing bone resorption, is initially unlabeled, and remains so for a time period, θ, required for the process of bone resorption to reach new bone formed after labeling of the pool. The terminology is essentially that of Robertson (5):

E = total amount of stable calcium in the miscible calcium pool, expressed in grams;
x_t = specific activity of the pool calcium, E, at a time, t, expressed as a fraction of the administered dose per gram of calcium;
k_u = fractional rate of loss of pool isotope by way of urinary excretion;
k_b = fractional rate of loss of pool isotope by way of new bone formation;
k_s = fractional rate of loss of pool isotope by way of fecal excretion;
K = fractional rate of loss of isotope by all routes from the pool, E;
θ = time required for the shortest of the long-period release mechanisms to return calcium^{45} to the pool, E.

If, for the sake of simplicity, the series of long-period delay cycles is arbitrarily

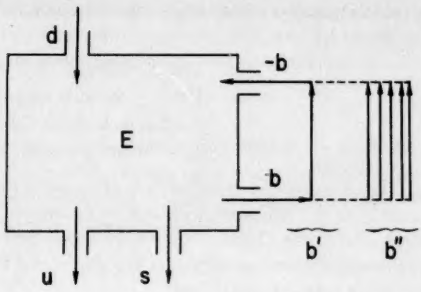

FIG. 1. Conceptual model employed in interpretation of C^{45} tracer data. See text for details.

divided into 2 subspecies—one cycle short enough to return isotope to the pool during the course of an average experiment (b'), and a series of cycles sufficiently long so that there is no return during an experiment (b'')—the rate of disappearance of isotope from the pool is given by the differential equation:

$$\frac{dx_t}{dt} = x_t(k_u + k_s + k_{b'} + k_{b''}) - k_{b'}x_{t-\theta} \qquad [1],$$

where the sum, $k_{b'}+k_{b''}=k_b$, and where these 2 constants represent fractional rates of loss of isotope into bone via the 2 subspecies of cycles just described. Reintroduction of additional delay cycles would require only further subdivision of k_b and the addition of appropriate terms to equation 1, and would not change the form of the equation.

For any time, $t \leqq \theta$, the term or terms representing delay cycles will be equal to zero, since recycling of isotope will not yet have occurred; and the specific activity of the pool will then be given by a simple exponential relationship:

$$x_t = x_0 e^{Kt}; \qquad t \leqq \theta \qquad [2];$$

and since as defined,

$$K = k_u + k_s + k_b \qquad [2a],$$

then

$$x_t = x_0 e^{(k_u+k_s+k_b)t}; \qquad t \leqq \theta \qquad [2b].$$

Such a formulation assumes instantaneous mixing, whereas, in fact, mixing within the system requires time, as is evident in the studies to be described and in the previously published work of Krane *et al.* (10), Bronner *et al.* (11), Spencer *et al.* (12), Armstrong *et al.* (13) and Thomas *et al.* (14). Completion of mixing in these studies, indicated by formation of a straight-line plot on semi-log coordinates, required periods ranging from twenty to forty hours. All the authors cited found their curves adequately described by equations with 3 or 4 decreasing exponential terms; but since for times which are long relative to mixing, all but the last of these terms reduce essentially to zero, and since the process of bone formation is not treated as entry of calcium into a *mixing* compartment, it seems much simpler and fully as accurate to employ an equation such as 2 or 2b, containing only 1 exponential term.

The actual generation of such an equation from experimental data gives rise to an exponential constant describing *isotope* behavior rather than a fractional-loss constant directly applicable to the pool calcium; but since, in an open system without internal recycling these 2 constants are equal in value (5), it is acceptable to use them interchangeably. This is also true for the fractional-loss constants, k_u, k_s, k_b, which properly

speaking apply directly to isotope movement. Thus rates of movement of stable calcium are given by:

Pool turnover	$= -KE$	[3],
Urinary calcium excretion	$= -k_uE$	[3a],
Endogenous fecal Ca excretion	$= -k_eE$	[3b],
New bone formation	$= -k_bE$	[3c].

The relationship which exists after recycling (that is for a time, $t > \theta$) is considerably more complex than that shown in equation 2. However, it can be demonstrated that after return via the shortest delay cycle, and prior to return through longer cycles, the new configuration assumed by the curve and the rate of bone formation via the returning cycle are related as follows:

$$k_{b'} = \frac{\frac{x_{2\theta}}{x_\theta} - \frac{x_\theta}{x_0}}{\theta} \qquad [4].^1$$

The importance of this relationship is that, under suitable circumstances, it becomes possible to calculate the portion of total bone formation which occurs by way of the shortest delay cycle.

Derivation of pool size

Pool size (E) is measured by extrapolating the exponential portion of the curve to zero time, and dividing this theoretical isotope concentration into the dose administered. This method neglects the excess urinary loss which unavoidably occurs prior to completion of mixing. On theoretical grounds this would seem to be acceptable, since urinary calcium in normal adults and most patients with bone disease is usually quite small relative to the total extracellular fluid calcium.

The relative importance of this excess excretion may be estimated by subtracting a theoretically calculated excretion (assuming instantaneous mixing) from the actually observed excretion during the first twenty-four hours. In the 12 patients reported in the present study, the magnitude of the excess was found to be only 1 per cent of the administered dose (range, 0.5–1.8 per cent). Whereas this excess may, in certain instances, represent a large fraction of the total urinary excretion during the first few days, it is not large enough to interfere with measurements of pool size. Thus, in these circumstances, it is valid to calculate the volume of the miscible pool by means of the usual equation:

$$E = \text{Dose}/x_0 \qquad [5],$$

the dimensions being units of weight of calcium.

Derivation of bone formation rate (BFR)

The rate of deposition of calcium in new bone (BFR) is derived from equation 3c,

[1] The explicit solution for equation 4, considering recycling through only one long-period delay cycle, assumes the form of a series of functions:

$$x_n(t) = \sum_{n=0}^{N} \frac{x_0(0)e^{K(t-n\theta)}[k_{b'}(t - n\theta)]^n}{n!}.$$

If this series be solved for the terminal point of the second period (2θ, $n=1$), equation 4, results. A detailed derivation of this expression may be obtained from the senior author on request.

E being calculated as described, and k_b being derived by difference from equation 2a. K can be measured experimentally, and the constants k_u and k_s, required for equation 2a, are derived from the usual relationship of precursor (pool) and product (excretion). Thus, for urinary loss,

$$k_u = K(f_{u_\infty}) \qquad [6],$$

where f_{u_∞} represents a theoretical asymptotic excretion value (expressed as a fraction of the total administered dose). In most cases this asymptote cannot be directly estimated, since equation 2b, upon which these calculations are based, is not valid at $t > \theta$. Thus f_{u_∞} must be calculated from excretory data obtained prior to θ, as follows:

$$f_{u_\infty} = \frac{f_{u_t}}{1 - e^{Kt}}; \qquad t \leqq \theta \qquad [7],$$

where f_{u_t} is the fraction of the administered dose excreted in the urine during the interval, 0 to t. This treatment is necessary because, whereas at $t \leqq \theta$ the 3 asymptotes are constants and their sum is equal to 1, at times when $t > \theta$, these asymptotes become functions of time: f_{b_∞} approaches zero, and the sum of f_{u_∞} and f_{s_∞} approaches 1.

Since, as indicated previously, excretory loss during mixing is always in excess of the theoretical, the measured value, f_{u_t}, will always be somewhat high. If relatively short time-intervals must be chosen, this excess excretion may introduce serious errors into the calculation of k_u. This difficulty can be avoided by excluding the mixing phase, using simultaneous equations of the same type as equation 7, and employing 2 values for t—one being after completion of mixing, and one at, or just prior to θ. Thus,

$$f_{u_\infty} = \frac{f_{u_{t_2 - t_1}}}{e^{Kt_1} - e^{Kt_2}} \qquad [7a].$$

These formulae may be combined, for the sake of simplicity, to yield:

$$\text{BFR} = EK(1 - f_{u_\infty} - f_{s_\infty}) \qquad [8],$$

in which the asymptotes are derived from equation 7 or 7a.

Experimental and analytical procedure

Calcium[45] was administered intravenously (as $CaCl_2$) from a calibrated syringe, in a single dose of 5–10 microcuries, to each of 12 adult subjects. One, Subject C.L., was a normal male with no significant disease, either of the skeleton or of other organ systems. The others had various types of metabolic bone disease, as indicated in Table 1. All patients were studied on the NIAMD Metabolism Ward, employing metabolic techniques previously described (15), and received constant weighed diets throughout each study. Each patient was subjected to the full metabolic and dietary routine for at least four days prior to injection of isotope, and in several instances had been receiving the constant diet for several weeks before the start of the tracer study. Effort was made to ensure a constant, moderate degree of physical activity throughout each of the studies; no patient was bedfast.

Urine was collected in 24-hour pooled specimens and feces in 4-day to 6-day periods. Serum samples were obtained at frequent intervals during the first two days of each study, and daily thereafter for seven to ten days. Stable calcium was determined by methods previously described (16), consisting essentially of permanganate titration of calcium oxalate. Urine samples for stable calcium were pre-digested with a mixture of nitric and perchloric acids. Calcium[45] was measured in aliquots of serum, undigested urine, and fecal ash. Counting was performed either in a gas-flow proportional counter, or with a thin end-window Geiger tube. Samples were counted on filter paper, as purified

calcium oxalate, each made up to a standard weight representing 5 mg. of calcium per planchette. The planchettes were of a special design described by Bloom (17).

A reagent blank was counted as a background sample, and known fractions of the administered dose were counted as standards. Sample density and counting geometry of the background and standards were identical with those of the unknowns. Counts were obtained to a standard error of ±3 per cent in all cases, except for a few serum samples drawn late in each study. The latter were counted to a standard error of ±5 per cent.

RESULTS

Table 1 lists the 12 patients studied, together with the diagnoses and pertinent observations. Graphs of the serum and urine calcium specific activity against time in 4 representative studies are presented in Figures 2–5. A portion of the bone formation data is also indicated graphically in Figure 6.

Except in the 2 patients with osteitis deformans, in whom the miscible calcium pool size was 398 and 438 mg. per Kg., the pool size in the remaining subjects (independent of diagnosis) ranged from 61–107 mg. of calcium per Kg. This range represents a total of between 4 and 8 grams of calcium in the average adult, and is less than 1 per cent of total body calcium in most of these subjects.

Bone formation rates (BFR) varied from a low value of 2.69 mg. Ca/Kg./day in a patient with untreated stable surgical hypoparathyroidism (and radiologically normal bone structure), to a high value of 154 mg. Ca/Kg./day in a patient with widespread osteitis deformans. The normal 65-year-old male had a BFR of 9.53 mg. Ca/Kg./day. Four patients with idiopathic and postmenopausal and/or senile osteoporosis, of varying severity, had BFR's ranging from 9.12 to 10.3 mg. Ca/Kg./day. Two postmenopausal women with osteoporosis of the axial skeleton together with chronic rheumatoid arthritis of peripheral joints had rates of 8.35 and 10.7 mg. Ca/Kg./day. A 27-year-old male with an extraordinary degree of generalized hyperostosis had a BFR of 16.2 mg. Ca/Kg./day. Two patients with generalized osteitis deformans had BFR's of 102 and 154 mg. Ca/Kg./day.

The times at which return through the shortest of the long-period release mechanisms was noted are also listed in Table 1. They range from approximately ninety hours in a patient with osteitis deformans, to over five hundred hours in a patient with hypoparathyroidism. The rate of bone formation through this cycle, when it could be measured (equation 4), represented from 20 to 40 per cent of the total bone formation rate. In the osteoporotic patients this value ranged from 1.6 to 2.7 mg. Ca/Kg./day. In the normal male (C.L.) the amount of bone formed which returned via this cycle was 3.2 mg. Ca/Kg./day, and in the 2 subjects with osteitis deformans, 31 and 65 mg. Ca/Kg./day.

TABLE 1. RESULTS OF CALCIUM BALANCE AND Ca^{45} TRACER STUDIES IN 12 PATIENTS

Subject	Age & Sex	Diagnosis	Ca intake (Gm./day)	Ca balance (Gm./day)	Duration of study (days)	E (mg. Ca/Kg.)	BFR (mg. Ca/Kg./day)	θ (hrs.)	Fraction of BFR returning at θ
C.L.	65 M	Normal control	1.13	−.010	22	93	9.53	150	.34
D.A.	40 F	Hypoparathyroidism (surgical)	.625	−.040	22	67	2.69	500	—
E.P.	62 F	Hypoparathyroidism (idiopathic); osteoporosis	1.72	+.070	23	68	6.29	245	.29
T.K.	65 F	Osteoporosis; rheumatoid arthritis	.400	−.021	18	61	8.35	150	.32
N.J.	46 F	Osteoporosis; rheumatoid arthritis	.406	−.20	22	63	10.7	150	.26
M.M.	42 M	Idiopathic osteoporosis	1.65	+.10	11	102	10.3	250	—
G.S.	51 F	Osteoporosis	1.44	−.22	23	95	10.0	200	.24
S.McL.	52 F	Osteoporosis	.678	+.026	22	79	8.12	320	.20
K.C.	55 F	Osteoporosis	.156	−.18	16	71	9.0	150	.24
E.C.	56 F	Osteitis deformans	1.02	−.010	16	398	154.0	93	.42
C.G.	63 M	Osteitis deformans	1.28	+.010	23	438	102.0	180	.30
J.P.	27 M	Familial hyperostosis	.431	+.035	16	107	16.2	240	—

DISCUSSION

A. *Evaluation of the model*

The assumptions and conditions under which the use of the proposed model may be valid should be clearly stated and an attempt made to justify such use. These assumptions are chiefly 2 (with several ramifications from each): 1) that the complexus of body calcium compartments behaves operationally as a single compartment, with 3 routes of loss; and 2) that the process of bone formation removes calcium from effective contact with the body fluids, and that bone resorption returns this calcium, without significant mixing with other bone calcium, after some minimum time period, θ.

Assumption of single compartment. The first assumption states that, whereas miscible body calcium exists anatomically in more or less distinct subcompartments, exchange between such compartments is sufficiently rapid so that ordinary physiologic loss from one compartment is immediately compensated by the much more rapid processes of mixing. Rough experimental confirmation of this assumption is obtained by comparing the final slopes of isotope disappearance curves with the slopes of the slowest "mixing" component (derived graphically in the usual fashion, by subtracting exponentials). Thus, in the series of Krane *et al.* (10) the average ratio of k_2 to k_3 (in the 13 studies described by 3 exponential terms) was 18.4/1 (range, 6.3/1 to 47/1); and in 11 of the presently reported 12 studies in which mixing data were available, the average ratio of the corresponding exponential constants was 13/1 (range, 7.9/1 to 23/1). It would appear that mixing is rapid relative to turnover, and that the system, despite its theoretical complexity, behaves operationally as a single compartment—at least under the conditions obtaining in the studies cited.

The same tentative conclusion can be reached by comparing available data on the rate of turnover of the system as a whole with independently determined exchange rates between various components of this system. The shortest turnover time ($1/K$) in the present series was approximately one hundred hours. Available estimates of blood calcium turnover times range from 1.1 to five minutes (4, 13, 14, 18), and exchangeable bone, muscle and tendon calcium have been shown to equilibrate with calcium in the bathing fluid in periods of one to five hours (19–21).

Such rates and times, although they are only approximations in some cases, and although they do not encompass all the possible intercompartmental exchanges, are nonetheless clearly very rapid relative to the turnover time of the system as a whole, and suggest that it is justifiable to treat the system of equilibrating body calcium compartments, from an operational viewpoint, as a unit whole.

Assumption of 3 routes of loss. Implicit also in the first assumption is the

adequacy of assigning only 1 route of loss from the pool for bone uptake. Whereas designation of urinary and fecal excretion as the first 2 routes raises no problems, radioactive calcium may be conceived as entering bone in at least 3 different ways: 1) rapid exchange with a small fraction of the bone calcium, 2) sequestration in the process of new bone formation, and 3) slow exchange between some major fraction of skeletal calcium and the relatively small miscible pool. The first mechanism is not a route of "loss" at all, since the rapidly exchangeable bone calcium is automatically included within the miscible pool. The second mechanism is incorporated into the proposed model as b and will be discussed subsequently. The importance of the third possibility, if it occurs, is that such loss of isotope into bone will be unaccompanied by net movement of stable calcium. That such a slow exchange may take place is suggested by the many published radioautographic studies, particularly those of Arnold *et al.* (19) and Cohen *et al.* (22). These authors, applying quantitative techniques to tibial-shaft bone found that a major fraction of the uptake in their specimens was accounted for by uptake into preformed mature bone. In some instances the rapidity of such uptake suggests that at least a portion of this bone calcium is included in the miscible pool and hence is not a "loss" from the pool.

Even so, it seems likely that a part of this uptake, although originally into exchangeable positions on the bone crystals, is eventually trapped by a process of "recrystallization," or solid-state diffusion. Theoretically this process would represent a very slow *exchange* between the miscible pool and the vast bulk of bone calcium; but because of the tremendous difference in size between these 2 slowly equilibrating compartments, the isotope movement over experimental periods measured in days would be essentially unidirectional (out of the miscible pool), and hence this process would appear to behave as an additional exponential route of loss from the pool. Thus any hypothetical system, such as the present model, which attributes the difference between turnover of pool isotope and loss of isotope by way of excretion, to the process of bone formation, must take into account the possibility of this slow sequestration of isotope by mature bone.

The studies just cited, since they deal with only a small segment of the total skeleton, and one composed principally of cortical bone, cannot furnish an accurate estimate of the overall significance of this exchange. It may, however, be possible to derive a rough upper limit for the process in question from an analysis of isotope data in conditions known to be associated with low rates of new bone formation, such as hypothyroidism and hypoparathyroidism. Patient D.A. in the present series, and Patients E.N. and W.W. (1), and B.M. (1) in the series of Krane *et al.* (see Tables 1 and 2) are examples. The rates of bone isotope uptake after mixing in these 4 subjects are roughly one quarter of that observed in normal subjects (see sub-

TABLE 2. BONE FORMATION RATES AND MISCIBLE CALCIUM POOLS RECALCULATED FROM PREVIOUSLY PUBLISHED STUDIES OF CALCIUM[45] METABOLISM IN MAN

Subject	Diagnosis	Age (yrs.)	E (mg.Ca/ Kg.)	BFR (mg.Ca/ Kg./day)
	From Krane, Brownell, Stanbury and Corrigan (10)			
L.S. [2]*	Hyperthyroidism, 4 mos. after surgery	31	121	17.2
Wa.W.*	Friedrich's ataxia	26	113	13.5
E.N.	Hypoparathyroidism	41	43	1.80
R.P.*	Arteriosclerotic heart disease	50	75	4.15
W.W. [1]	Myxedema	42	69	1.93
W.W. [2]*	Myxedema, post-treatment		78	7.46
B.M. [1]	Myxedema	39	57	2.59
B.M. [2]*	Myxedema, post-treatment		85	10.7
F.W. [2]	Hypoparathyroidism, post-surgical	19	118	31.3
	From Bauer, Carlsson and Lindquist (7)			
17*	Cerebral tumor	25	77	7.7
20	Leukemia	22	114	12.9
21*	Cerebral tumor	26	75	6.7
22	Acute myeloid leukemia	43	80	6.3
23*	Cerebral tumor	51	79	5.6
24	Chronic granulocytic leukemia	56	70	9.7
25	Pituitary tumor (resected)	57	68	4.8
26	Esophageal cancer (with obstruction)	58	62	7.0
27*	Cerebral tumor	60	66	8.7
28	Lymphatic leukemia	63	88	5.1

* Probably normal with respect to bone metabolism—on the basis of available clinical data.

sequent section). If this uptake were totally by virtue of slow exchange, and if the rate of this exchange were unaltered by the disease process, then at least 75 per cent of bone uptake in subjects with normal or elevated rates could be attributed to actual bone formation. Since even this estimate assumes complete cessation of new bone formation in these disorders, and since this is improbable, it follows that the degree of overestimation at normal rates is probably much less than 25 per cent.

The bone formation rates described in this paper, as well as those published by Bauer and co-workers (7), involve, for simplicity, the assumption that total bone uptake (exclusive of that included in the pool) represents new bone formation. Nonetheless the uncertainty attendant upon this matter imposes obvious and important restrictions on the measurement of such rates.

Assumption that bone formation removes calcium from the pool. The second

assumption upon which the use of the proposed model is based is that calcium deposited in new bone at the time of its formation becomes isolated from the pool, and furthermore is not exchanged significantly with adjacent bone calcium. A large number of radioautographic studies (of which those of Comar *et al.* (23) Amprino (24), Leblond *et al.* (25), and Arnold *et al.* (19) are representative) indicate clearly, at least in a semiquantitative way, that this is true. These studies have demonstrated that a layer of bone deposited either in a forming Haversian system, or on the surface of a bone trabecula, retains throughout its biologic life the specific activity given to it at the time of its formation by the pool from which it was formed. This means that such bone is effectively isolated both from the pool and from adjacent bone calcium, and that such bone at the time of its destruction returns calcium to the pool at an isotope concentration which had existed in the pool at some earlier time. Recent studies of Hevesy (26) provide additional support for this concept. This investigator, employing newborn rats with uniformly labeled skeletons, maintained for various times on diets free of Ca^{45}, demonstrated that major fractions of the animals' life-span were required for mobilization of deeply situated isotope, suggesting that such mobilization must be principally by structural revision of bone.

The conclusions flowing from these observations are in opposition to earlier views concerning the extensive exchangeability of bone mineral (27–29). This conflict is resolved by Neuman and Neuman (1, 30), who point out that, because of a virtual absence of free water in mature bone, newly-formed bone retains contact with extracellular fluid for only a relatively short time after its formation.

Characteristics of recycling time. Implicit also in the second assumption is the crucial matter that θ, the time required for recycling of isotope through the shortest-lived species of bone, is long relative to mixing time. The importance of this point is that the occurrence of such recycling within, or only shortly after mixing would make impossible the experimental measurement of K, upon which the remainder of the calculations rests. Very little experimental evidence bears on this matter. The data of Carlsson (31) would indicate a minimum interval of eighteen to twenty-four hours before calcium is resorbed from the femoral epiphysis of a growing rat, and the work of Leblond and associates (25) demonstrated a maximum specific activity of the epiphyseal plates of adult rats at approximately forty-eight hours. Evidence for the human subject is lacking, but in view of the delay time manifested in the most rapidly turning-over centers of bone formation in growing small animals, it is probable that at least several days would elapse in an adult human subject between bone deposition and its subsequent dissolution.

In this regard, in any system such as the proposed model, at $t=\theta$, the slope of the isotope disappearance curve will be more or less abruptly altered. Available data conform to this prediction: such a break was observed in 10 of the 12 patients in the present series, and has been observed repeatedly by Bauer *et al.* (7, 9).

An important question, however, is whether the observed points prior to $t=\theta$ in reality approximate a straight line on semi-log coordinates. This condition must be met to permit both accurate calculation of K and localization of θ. Such straight lines are indicated in Figures 2–5, and it would appear in most cases that these lines are at least as plausible as any alterna-

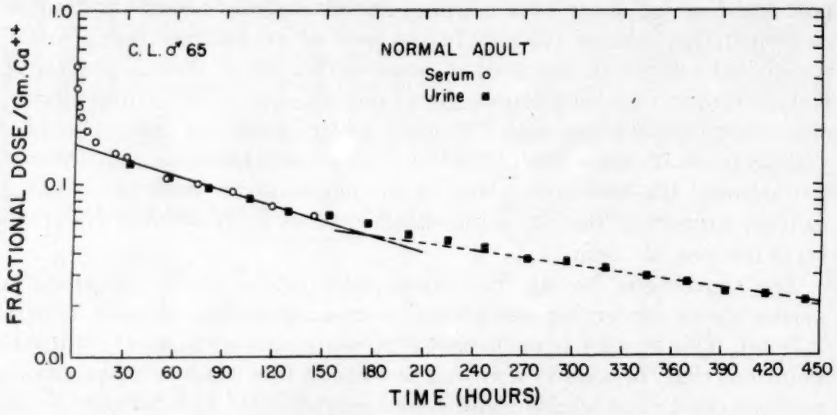

FIG. 2. Semilogarithmic plot of serum and urine specific activity in normal 65-year-old male subject (C.L.).

tive construction. Similar exponential curves, commencing at approximately twenty to forty hours after injection of the isotope, have been observed in the previously cited studies of Ca^{45} behavior in both man and animals (4, 7–10, 13, 14). It is concluded, therefore, that the exponential constant, K, describing the fall in pool isotope concentration after mixing and prior to θ, adequately describes the turnover of the system as a unit whole.

In osteitis deformans, however, and indeed in any situation in which θ is relatively small (such as may perhaps occur in hyperthyroidism and hyperparathyroidism), the assumption of a straight line prior to θ is not as convincing as when θ is long, and the precise localization of θ itself may be uncertain. Thus, estimates of bone formation rate under such circumstances must be somewhat cruder approximations than can be made when K may be obtained with considerable accuracy.

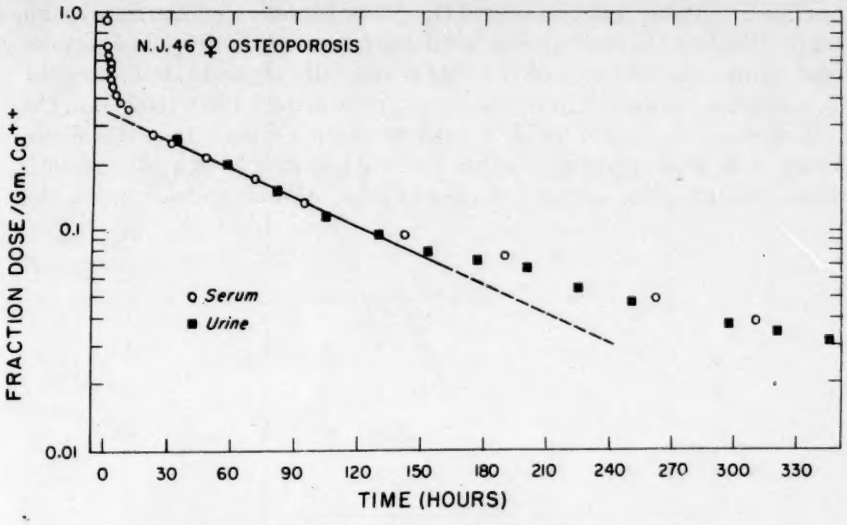

FIG. 3. Semilogarithmic plot of serum and urine specific activity in 46-year-old female (N.J.) with postmenopausal osteoporosis.

B. *Miscible calcium pool*

The measured pools in most of the subjects ranged from 60 to 110 mg. of calcium per Kg. of total body weight. Pools of this magnitude have been

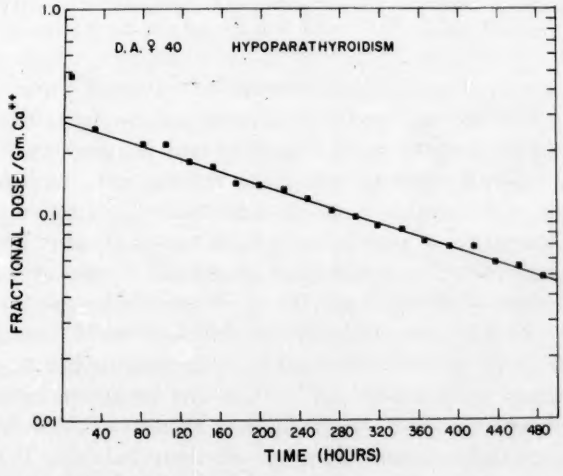

FIG. 4. Semilogarithmic plot of urine specific activity in 40-year-old female (D.A.) with untreated surgical hypoparathyroidism.

described by Bauer and co-workers (7, 9) and Bronner and Harris (4), and can be calculated from the published data of Krane *et al.* (10). The physiologic significance of pools of this size is not fully apparent, but since the total amount of calcium in the body is approximately 1200–1500 Gm., the bulk of skeletal calcium must be exterior to such a pool. Indeed, the observed sizes correspond reasonably well with available estimates of soft-tissue calcium. The excellent studies of Mitchell and co-workers on ele-

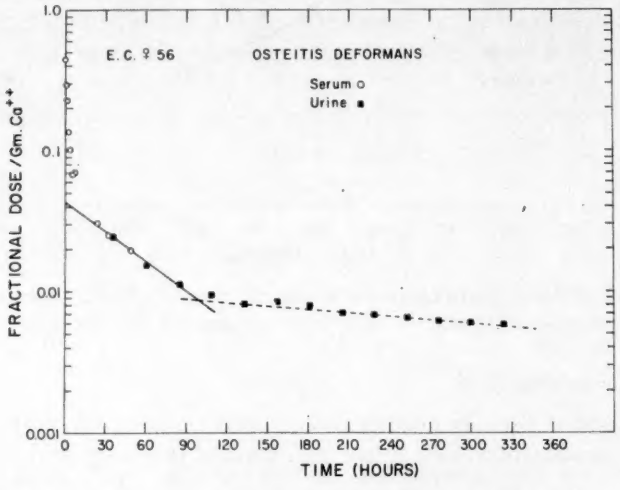

FIG. 5. Semilogarithmic plot of serum and urine specific activity in 56-year-old female (E.C.) with widespread osteitis deformans.

mental composition of human cadavers (32–34) revealed a mean soft-tissue calcium content of 155 mg. per Kg. of total body weight. The difference between this figure and the pools described here suggests that perhaps as much as 50 per cent of the total soft-tissue calcium detectable by chemical analysis is not exchangeable with an intravenously administered tracer. This non-exchangeable soft-tissue calcium is most probably accounted for by insoluble calcific deposits attendant upon local disease or degeneration. Such a conclusion is strengthened by a review of the published data of these workers, which reveals that tissues labeled "solid remainder," constituting from 4–11 per cent of total body weight in the 4 subjects analyzed, contained from 34–53 per cent of the total soft-tissue calcium.

The miscible calcium pool, on the basis of these studies, would therefore seem to be adequately accounted for by soft-tissue calcium. This, however, cannot altogether be true, since the radioautographic studies previously cited demonstrate clearly that some fraction of bone calcium is exchanged rapidly with body-fluid calcium. Newly formed immature bone certainly

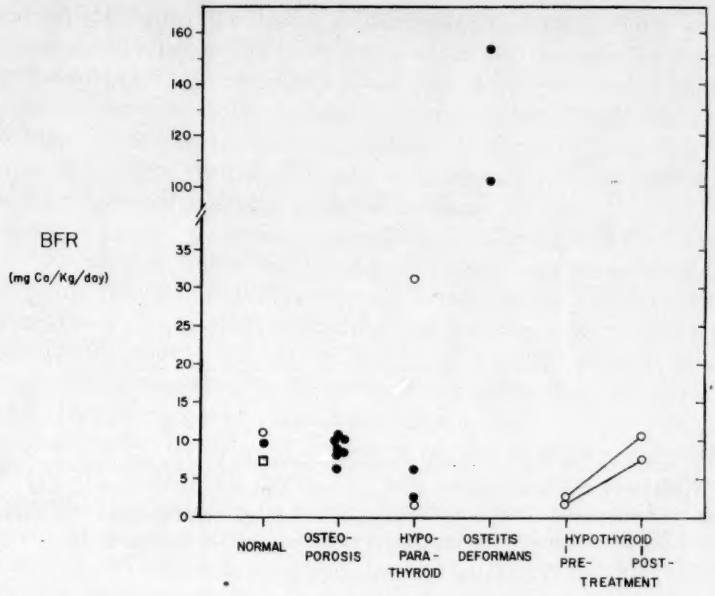

FIG. 6. Graphic representation of bone formation rates (BFR) in normal subjects and in patients with selected metabolic and bone diseases. Solid circles (●) represent data from the present study; open circles (○), from the study of Krane *et al.* (10); and open square (□), from the study of Bauer *et al.* (7). The points in the "normal" column represent means of the values for normal subjects recalculated from the data of these workers (see Table 2).

participates in this exchange (1,24,25), and it is probable that a small fraction of mature bone is also involved (19,22).

It appears reasonable to conclude, therefore, that the "miscible calcium pool," as determined by tracer methods, is composed principally of soft-tissue soluble calcium, together with the variable and usually small contribution of exchangeable bone calcium.

Since isolation and maturation of bone requires time, and since immature bone contributes to the miscible pool, it is to be anticipated that high BFR's would be associated with large pools. This relationship has been discussed by Bauer, Carlsson and Lindquist (35) and may be invoked to explain the large pools observed in the present series in the 2 patients with osteitis deformans. Figure 7, in which BFR's are plotted against pool sizes, indicates the close relationship which exists between these 2 variables.

C. *Formation of short-lived bone*

The available data are too meager to allow definite conclusions concerning the recycling of isotope through a short-lived species of bone. It is of

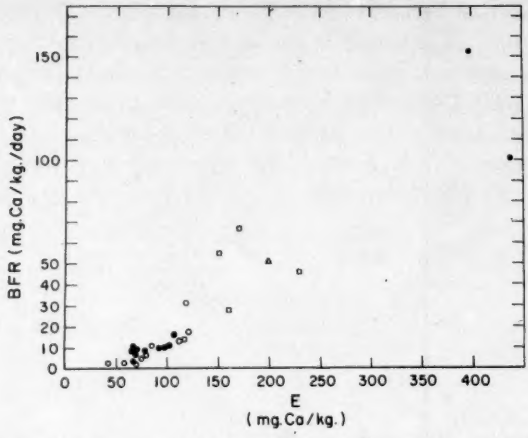

FIG. 7. Relationship between bone formation rate (BFR) and miscible calcium pool (E) in 35 subjects with and without bone disease. Explanation of the symbols is given in the legend for Figure 6. The open triangle (△) represents a mean value for Subjects Nos. 10–18 and 55, normalized for weight, from the study of Bronner and Harris (4). Coefficient of correlation = +0.94.

interest, however, that the shortest such life was observed in Patient E.C with osteitis deformans, and that Patient D.A. with uncomplicated hypoparathyroidism demonstrated no such recycling to the termination of the study (five hundred hours). These observations seem compatible with the more or less obvious inference that the time-lag between bone formation and its subsequent resorption will vary inversely with the rate of bone turnover.

It is noteworthy that the calculation of this recycling indicates that 20 to 40 per cent of total new bone formation in the present studies was devoted to construction of bone elements with a relatively short life. The structural value of such bone may perhaps be questioned, and it is tempting to speculate that this continuously renewed and renewable bone, with its structure probably never reaching full maturity, and its calcium thus still in relatively intimate contact with body fluids, may serve as one of the principal mechanisms by which changes in extracellular fluid calcium are buffered.

D. *Rate of bone formation*

Evaluation of the observed BFR's in various disease states depends heavily on knowledge of the normal BFR. The fact that there was only 1 normal subject in the present series is disadvantageous, but we are helped somewhat by review of the presently available studies of Ca^{45} metabolism

in man. On the basis of published clinical data, 9 subjects who were probably normal with respect to bone metabolism can be selected from the series of Bauer *et al.* (7) and Krane *et al.* (10). BFR values and pool sizes recalculated from the data of these workers are listed in Table 2. It is realized that those patients selected as normal may not be altogether representative of the true normal, since the mere absence of bone disease in itself does not guarantee normal bone metabolism.

With this reservation in mind, the mean values for BFR in the "normal" group of Krane *et al.* is 10.6 mg. Ca/Kg./day, and from the series of Bauer *et al.*, 7.2 mg. Ca/Kg./day. The only definitely normal subject (C.L.) in the present series had a BFR of 9.53 mg. Ca/Kg./day. The mean for all 10 subjects was 9.1 mg. Ca/Kg./day (S.D., ±3.9). Further study is indicated, both to establish more specifically the upper and lower limits of normal and to assess changes that may occur with aging.

The observations on the rate of bone formation in untreated hypoparathyroidism are of interest. Since it is now established that parathyroid hormone directly stimulates or at least facilitates bone resorption (36, 37), a decrease in bone turnover (and hence in bone formation) is to be anticipated in hypoparathyroidism. The rates in Patients D.A. (2.69 mg. Ca/Kg./day) in the present series, and Patient E.N. (1.80 mg. Ca/Kg./day) in the series of Krane *et al.*, seem clearly lower than those of the normal subjects, and are thus in accord with theory. Patients E.P. (6.29 mg. Ca/Kg./day) and F.W.-2 (31.3 mg. Ca/Kg./day) of Krane *et al.* are exceptions. Patient E.P. had coexisting marked osteoporosis, and it is tempting to speculate that increased mechanical stress per unit of bone mass may have provided a stimulus to bone formation. Patient F.W.-2 in Krane's series was an adolescent and still presumably in a period of bone growth. The treatment status is uncertain, but a higher rate of bone formation than that of a normal adult is to be anticipated in a patient of this age. The fact that such was observed, even in the face of hypoparathyroidism, serves as indirect clinical confirmation of the view advanced by Talmadge and others (38) that parathyroid activity merely regulates the extracellular fluid calcium level at which homeostatic and metabolic processes occur and does not, itself, determine these processes.

The recalculated values from the studies of Krane *et al.* on 2 patients with hypothyroidism before and after treatment (W.W. and B.M.) are presented here for the first time, because they corroborate the long held view that bone metabolism is reduced in hypothyroidism. In both these untreated patients the rates of bone formation were low—1.92 and 2.59 mg. Ca/Kg./day, respectively. With thyroid replacement therapy there was a fourfold increase in these values.

Patient J.P. in the present series, with severe diffuse hyperostosis, dem-

onstrated an apparently high rate of bone formation. A higher than normal rate is consistent with the dense bone structure exhibited by this patient, but until the upper limit of normal can be better established the significance of values in this range will remain unsettled.

Patients E.C. and C.G. in the present series, both with osteitis deformans, demonstrated the extraordinarily high rates of bone formation found in this disorder. These rates (154 and 102 mg. Ca/Kg./day), when translated into terms of whole bone, are equivalent to between 80 and 90 grams of new bone formed per day. They are in accord with existing concepts of the rate of bone formation in this disorder, and would be anticipated from the degree of hypercalcemia and hypercalciuria precipitated by immobilization in these patients (39).

Perhaps the most striking observations in the present series is the level of bone formation in the 7 patients with osteoporosis. The mean for this group was 8.7 mg. Ca/Kg./day. If Patient E.P. be excluded, because of the complicating influences of her hypoparathyroidism (serum calcium level about 7 mg. per 100 ml.), the mean rate of bone formation for the remaining 6 subjects is 9.4 mg. Ca/Kg./day. Either value is clearly not significantly different from available data on normal subjects. The 6 studies in patients with osteoporosis uncomplicated by any evident alteration in parathyroid function were carried out at a wide variety of calcium intake levels. In 4 of the patients there was a negative calcium balance, and in 2 there was a positive balance; yet the BFR values were closely similar (S.D. ± 1.07 mg. Ca/Kg./day). Thus it is difficult to support a suggestion that the disease may have been inactive in any of these studies. More important, the finding of "normal" values for BFR in these patients suggests the possibility that the diminished bone mass in osteoporosis may be associated, not with decreased bone formation, but with elevated bone destruction.

This suggestion clearly runs counter to existing concepts of the nature of osteoporosis (40–42). However, such concepts, based principally upon the many careful observations by Albright and his co-workers, have never been subjected to definitive kinetic analysis. The only information thus far available on the rate of bone formation in osteoporosis is found in recent studies by Bauer, Carlsson and Lindquist (43) and by Eisenberg, Fraser and Gordan (44). Both studies reported definitely reduced BFR's in patients with senile osteoporosis. The discrepancy between these results and those reported here cannot immediately be resolved, but it should be pointed out that there are valid methodologic objections to both of these studies. Bauer and co-workers employed tibial-bone biopsy specimens and measured local P^{32} uptake, rather than total skeletal uptake; it is not certain that such bone can be taken as representative of total skeletal activity.

Eisenberg and co-workers, on the other hand, measured the total skeletal uptake of strontium, but carried out their studies for only four days; in most cases K cannot be calculated with sufficient accuracy within this time to allow fine comparison between studies.

Albright and associates have maintained that quantitatively deficient protein matrix formation represents the sole defect in osteoporosis. The observation that bone formation is not lower than normal in this disorder, as well as accumulating evidence implicating insufficient dietary calcium intake (45–48), suggests that the Albright hypothesis may prove to be inadequate.

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ESTROGEN EXCRETION IN OVARIECTOMIZED WOMEN RECEIVING ADRENOCORTICOTROPIN*†

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ABSTRACT

Increased urinary estrogen excretion was observed in each of 9 ovariectomized but otherwise normal women receiving large doses of ACTH for three days. Increases were modest, reaching only the lower limits of the range for normal women of reproductive age, even when treatment was prolonged for thirteen days. No increase in estrogen excretion occurred in 3 women receiving large doses of hydrocortisone. It is concluded that the enhanced estrogen excretion occurring in women receiving ACTH is due to direct stimulation of adrenal estrogen excretion rather than a conversion of adrenal steroids to estrogen or an effect of the adrenal steroids on the kidney causing enhanced excretion of estrogens.

THE human adrenal cortex is known to be capable of producing estrogens. In adrenocortical carcinoma (1) large amounts of estrogens can be recovered from the urine and, clinically, feminization is seen. Moderately elevated estrogen levels have been found in congenital adrenal hyperplasia (2). Dao (3), Huggins (4), and West and co-workers (5), in studies on women with breast cancer, detected estrogens in the urine after bilateral ovariectomy, but not after combined ovariectomy-adrenalectomy. The latter investigators, as well as Nathanson and associates (6) and Paschkis (7), showed that stimulation of the adrenal cortex with adrenocorticotropin (ACTH) in various neoplastic diseases may increase the excretion of urinary estrogens. Conversely, treatment of congenital adrenal hyperplasia by suppressing endogenous ACTH secretion with cortisone decreases the initially high estrogen levels found in this condition (2, 8, 9).

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The foregoing investigators were concerned with the adrenal responses in certain diseased states. The purpose of the present studies was to determine the ability of the adrenal cortex to secrete estrogens under normal conditions and during the administration of ACTH.

MATERIALS AND METHODS

Patients. Studies were made in 9 ovariectomized women, 35 to 52 years of age. Ovariectomy, incidental to pelvic surgery for pelvic inflammatory disease or uterine leiomyomata, had been performed at least one year previously. Each patient presented with typical menopausal symptoms, but none had received estrogen therapy for at least six months prior to this study.

Treatment. After urine for control studies had been collected, ACTH gel[1] was administered intramuscularly once daily for three days in doses of 200, 100, and 100 units, respectively. Patient T.P. received ACTH gel for thirteen days—200 units the first day and then 120 units once daily for twelve days. Hydrocortisone was administered orally in two equal doses for three days to 2 ovariectomized patients and 1 normal postmenopausal woman. A dose of 100 mg. was given the first day, and 50 mg. on each of the two subsequent days.

Methods. Before treatment, two 24-hour urine specimens were collected by each patient on two occasions, approximately one week apart. Then 24-hour urine specimens were collected on the second and third days of ACTH and hydrocortisone administration. In Patient T.P., who received ACTH for thirteen days, studies were performed at one-day to two-day intervals during treatment, and seven days after treatment.

Urinary estrogens were assayed biologically by a method previously described (10). In this method the sodium hydroxide-soluble fraction of the chloroform extract of acid-treated urine is administered to immature female rats. Increases in uterine weight produced by two or more dilutions of urine extract are compared with responses produced by estradiol benzoate. The results are expressed as microgram-equivalents of estradiol benzoate per 24-hour urine specimen.

Urinary 17-ketosteroids were determined by the method of Drekter and co-workers (11).

RESULTS

Urinary estrogen excretion averaged 0.2 μg. per twenty-four hours before treatment. During ACTH therapy, estrogen excretion increased in each patient, averaging 0.6 μg. per twenty-four hours on the second and third days of administration (Table 1). A substantial increase in 17-ketosteroid (17-KS) excretion was observed in each subject, with the exception of Patient C.B., in whom 24-hour 17-KS excretion increased only from a pretreatment average of 8.1 mg. to 11.6 mg. on the third day of ACTH administration.

The greatest increases in the urinary excretion of estrogens and 17-KS were noted in Patient T.P., who received ACTH for thirteen days (Fig. 1).

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[1] HP ACTHAR® gel, generously supplied by the Armour Laboratories, Chicago, Illinois.

TABLE 1. URINARY ESTROGEN AND KETOSTEROID EXCRETION IN 9 OVARIECTOMIZED WOMEN RECEIVING 200, 100, AND 100 UNITS OF ACTH ON EACH OF THREE SUCCESSIVE DAYS

Patient	Age (yrs.)	Urinary estrogens (μg. per 24 hrs.)			Urinary 17-ketosteroids (mg. per 24 hrs.)		
		Control	During ACTH administration		Control	During ACTH administration	
			Day 2	Day 3		Day 2	Day 3
L.H.	47	<0.2 0.2	0.5	0.6	5.2 9.1	21.9	22.6
S.G.	52	<0.2 0.3	0.8	0.7	3.9 2.8	12.1	10.8
J.J.	43	0.2 0.4	0.8	0.6	8.9 4.2	21.3	17.7
F.G.	43	0.3 0.2	0.6	0.6	7.7 6.5	8.3	13.0
V.M.	49	<0.2 0.3	0.4	0.8	6.4 9.1	15.4	14.6
K.B.	35	<0.2 <0.2	0.7	0.7	7.2 6.9	13.4	18.6
C.B.	40	0.3 0.2	0.4	0.4	8.9 7.3	10.2	11.6
F.W.	41	<0.2 0.3	0.4	0.4	12.5 14.5	19.5	26.3
T.P.	35	0.2 <0.2	0.5	0.5	8.2 7.2	21.0	21.1
Average		0.2	0.6	0.6	5.9	15.9	17.4

Peak levels were reached on the seventh day of administration, being 0.9 μg. per twenty-four hours for estrogens and 36.7 mg. per twenty-four hours for 17-KS. Six days after discontinuing ACTH, 17-KS and estrogen excretions were at pretreatment control levels.

No increase in urinary estrogen excretion occurred in the 3 patients receiving hydrocortisone for three days (Table 2).

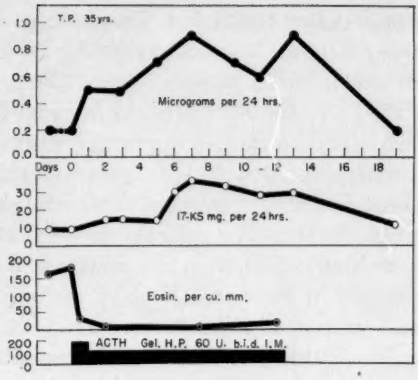

FIG. 1. Urinary estrogen excretion in a 35-year-old ovariectomized woman receiving ACTH gel intramuscularly for thirteen days—200 units the first day and then 120 units for twelve days. Urinary estrogens are expressed as micrograms of estradiol benzoate per 24-hour urine specimen.

DISCUSSION

Our studies demonstrate that the normal adrenal cortex responds to stimulation with ACTH by secreting an increased amount of estrogen. This is in keeping with the findings of Nathanson and co-workers (6) and West and co-workers (5), who observed increased excretion of estrogen in cancerous patients who were receiving ACTH. The increases in estrogen excretion observed in our patients were not as great as those produced by West in cancerous patients. However, strict comparison with the latter study is not possible, since details concerning the amount of ACTH and the duration of administration were not included.

Urinary estrogen excretion was uniformly low in the ovariectomized pa-

TABLE 2. URINARY ESTROGEN EXCRETION IN WOMEN RECEIVING 100, 50, AND 50 MILLIGRAMS OF HYDROCORTISONE ON EACH OF THREE SUCCESSIVE DAYS

Patient	Age (yrs.)	Urinary estrogen (μg. per 24 hrs.)		
		Control	During hydrocortisone administration	
			Day 2	Day 3
L.P. (castrate)	45	<0.2 0.2	<0.2	<0.2
A.W. (castrate)	44	0.2 0.2	0.2	<0.2
M.D. (postmenopausal)	56	0.3 0.2	—	<0.2

tients before treatment. The average value of 0.2 μg. per twenty-four hours was comparable to that found by Paulsen and associates in a larger series of ovariectomized patients (12). The increase in production of estrogen induced by stimulation of the normal adrenal cortex with ACTH appeared definitely limited, the average during treatment being only 0.6 μg. per twenty-four hours. The responses may have been limited in part by the short period of treatment (three days). However, in the patient treated for thirteen days, the greatest increase amounted to only 0.9 μg. per twenty-four hours. Although the response was definitely limited, the peak levels reached in Patient T.P. were within the lower limits of our range for normal women of reproductive age (12).

The studies of Heller and co-workers (13) suggest that the increased secretion of ACTH induced by stress may result in clinically recognizable changes due to enhanced estrogen output by the adrenal cortex. They observed transient estrogenic effects in the vaginal cells following abdominal surgery. The time relationship suggested that these changes were due to the stress of operation, which in turn resulted in stimulation of the adrenal cortex.

It is possible that the increased excretion of estrogen seen in our patients receiving ACTH was due to conversion of adrenal steroids to estrogen, or an effect of adrenal steroids on the kidney causing enhanced excretion of estrogen. Neither of these possibilities seems likely, since no increase in estrogen excretion was observed in the 3 patients receiving large doses of hydrocortisone. Furthermore, in men receiving large doses of cortisone, urinary estrogen excretion is not increased (14); actually, slight decreases are observed, suggesting inhibition of adrenal estrogen production by the administration of cortisone.

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A SIMPLIFIED METHOD FOR THE ROUTINE MEASUREMENT OF URINARY ESTRIOL*

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ABSTRACT

A method for the measurement of urinary estriol, suitable for the hospital laboratory, is described. It involves enzymatic hydrolysis of urinary estriol glucuronide, ether extraction, rapid saponification, simplified column chromatography, and fluorometry. The specificity of the assay has been checked by comparing the amount of estriol so obtained with the amount of estriol methyl ether isolated from the same extract of urine after methylation and re-chromatography. The method has been shown to be sensitive and accurate by the performance of recovery experiments with estriol glucuronide added to urine and by means of duplicate analyses of urine, which agree within 10 per cent. Assay results correlate well with cyclic changes in the secretory function of the ovaries during the menstrual cycle, and with other measurements of adrenocortical hyperfunction in patients with congenital adrenal hyperplasia. The method can detect increased estrogen secretion by the adrenal cortex in response to the administration of ACTH.

THE methods described by Brown (1) and by Bauld (2) for the chromatographic separation and individual assay of the principal estrogens in urine—estrone, estradiol and estriol—are largely restricted to research laboratory use because of their complexity. The need for an equally reliable but simpler method, suitable for the hospital laboratory, has prompted the development of the procedure reported in this paper.

It was reasoned that measurement of urinary estriol alone should prove adequate for clinical purposes, since the ratio of one estrogen to another in the urine has been reported to vary surprisingly little in normal and disease states (3); and in the case of 2 known exceptions, pregnancy (4) and the neonatal period (5), large amounts of estriol are found, rather than estrone or estradiol. A recent study (6) has verified the logic of this line of reasoning by demonstrating that estriol excretion correlates well with biologic estimation of estrogen stimulation in normal subjects, and in this

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respect is a more reliable index than the total urinary estrogen level or the amount of estrone or estradiol excreted.

It was felt that certain methodologic advantages might be gained by limiting the assay to estriol. The conditions of hydrolysis, extraction, purification and measurement could be more rigidly standardized for a single steroid than for a mixture of three (7). Since estriol is almost invariably excreted in substantially greater amount than the other two estrogens (3, 4), only small volumes of urine and solvents would need be handled. The substitution of fluorometry for colorimetry should increase the sensitivity of the assay.

The procedure finally devised and subjected to critical evaluation involves enzymatic hydrolysis of urinary estriol glucuronide, extraction of the liberated estriol, rapid saponification, simplified column chromatography, and fluorometry. This is in effect a simplified variant of the basic procedure reported by Bauld, and appears to yield equally valid results. The relative simplicity of the procedure enables one technician to assay 6 to 8 specimens of urine in a six-hour working day.

MATERIALS AND METHODS

All glassware is carefully washed with Alconox (Harshaw), copiously rinsed first with tap water, then distilled water, and allowed to dry at room temperature. Each item must be carefully inspected for cleanness before use.

Apparatus and reagents

1. Sodium phosphate buffer, 0.75 *M*, pH 6.5.
2. Bacterial glucuronidase (Sigma Chemical Co.). The solution, containing 2500 units per milliliter, may be stored in the refrigerator for at least four months.
3. Ethyl ether, reagent grade (Merck), redistilled over anhydrous sodium carbonate.
4. "Concentrated carbonate solution" of Bauld (2): 130 ml. of 5.0 *N* NaOH diluted to 1 liter with 1.0 *M* $NaHCO_3$.
5. Serum bottles, 100-ml. capacity, with inset rubber stoppers.
6. Pressure cooker (Presto Model 104), 4-quart capacity.
7. Concentrated HCl, reagent grade (Baker).
8. Syringes, 5-ml. capacity, fitted with 1½-inch 22-gauge needles.
9. Alumina powder, activated catalyst grade (Harshaw Chemical Co.). Water content 3.6–3.8 per cent.
10. Benzene, reagent grade (Merck), redistilled over anhydrous sodium carbonate.
11. Ethanol, absolute (Gold Shield Label, Commercial Solvents Corp.).
12. Sulfuric acid, reagent grade (Mallinckrodt); 90-vol.% and 65-vol.% solutions are made with distilled water and discarded after one month.

Hydrolysis and extraction

A 20-ml. aliquot of urine from a 24-hour collection, 2.0 ml. of phosphate buffer, and 0.2 ml. (500 units) of glucuronidase solution are incubated at 37° C. overnight (sixteen to eighteen hours). The mixture is extracted twice with 15 ml. of ether in a small separatory funnel and the combined ether extracts are washed once with 5 ml. of the

concentrated carbonate solution diluted to 10 ml. with distilled water. The ether is collected into a serum bottle and evaporated at 45° C. with a stream of filtered air. Distilled water is added to the pressure cooker and brought to boiling on a hot plate. The dried crude extract in the serum bottle is dissolved in 10 ml. of 1.0 *N* NaOH. The bottle is stoppered and a 22-gauge needle inserted through the stopper. The alkaline solution is autoclaved at 10 pounds pressure for five minutes; this is carried out by removing the pressure cooker from the hot plate when the pressure rises to 10 pounds and allowing it to sit for five minutes at room temperature. The temperature is then rapidly lowered by running cold tap water over the lid of the pressure cooker. The bottle is removed and rapidly cooled to room temperature by setting it in a dish or tray of cold water. The contents are transferred to a small separatory funnel and the bottle is rinsed with 15 ml. of ether, which is used to extract the alkaline solution and then discarded. To the aqueous layer in the separatory funnel is added 1.0 ml. of concentrated HCl. The acidified solution is extracted twice with 15 ml. of ether. The aqueous layer is discarded. The ether layers are combined and washed with 12 ml. of undiluted carbonate solution, by the procedure of Bauld (2). After discarding the aqueous layer, the ether is washed with 3 ml. of 2 *N* NaOH. Both layers are left in the funnel. After the addition of 12 ml. of 1.0 *M* $NaHCO_3$, the mixture is shaken and the aqueous phase is discarded. The ether is finally washed with 1.5 ml. of distilled water, collected into a 100-ml. flask, and evaporated with a stream of air at 45° C.

Column chromatography

The barrel of a 5-ml. syringe, fitted with a 22-gauge needle, serves as a satisfactory column. The bottom hole is covered with a small plug of washed glass wool, 1.0 gram alumina is added, and a second glass-wool plug is inserted to protect the top of the column. The syringe is loosely inserted into an 18×150 mm. test tube and moved from one test tube to the next as the various fractions are collected. The column is washed initially with 10 ml. of a 2 per cent solution (v/v) of ethanol in benzene. The saponified extract is transferred to the column by rinsing the flask containing it with five 2-ml. portions of the same solvent. The column is washed with 5 ml. of a 4 per cent solution of ethanol in benzene, which is discarded. The estriol fraction is eluted with 15 ml. of a 30 per cent solution of ethanol in benzene and dried at a temperature of 45–50° C. under a stream of filtered air.

Fluorometry and calculations

The chromatographed dry residue, which should be colorless, is dissolved in 0.3 ml. of absolute ethanol, and 0.1 ml. is transferred to each of two 18×150 mm. Pyrex test tubes. One aliquot is dried and saved for subsequent analysis if the original fluorometric reading is found to be too high. To the second tube is added 1.0 ml. of 90 per cent sulfuric acid. Two "blanks" are prepared. The first (acid blank) contains 0.1 ml. of ethanol and 1.0 ml. of 90 per cent sulfuric acid; the second (solvent blank) contains one-third of the residue obtained by evaporating 15 ml. of a 30 per cent solution of ethanol in benzene, 0.1 ml. of ethanol, and 1.0 ml. of 90 per cent sulfuric acid.[1] Two estriol standards, 0.2 and 0.4 microgram, are run simultaneously in duplicate. The tubes are heated in a boiling-water bath for five minutes and allowed to cool at room temperature for five to ten minutes. To each tube is added 2.0 ml. of 65 per cent sulfuric acid, and the contents are mixed. The contents are transferred to small test tubes and read with a

[1] This blank was found to be identical with a 30 per cent ethanol/benzene fraction from the alumina column.

Farrand Model A fluorometer. A combination of Corning filters 3384, 4784 and 5113 is used as the primary filter, and Corning filter 3385 as the secondary filter. The sensitivity is set so that the 0.2-microgram standard reads between 90 and 95 on the galvanometer scale, and at this sensitivity the unknown as well as the blanks are read.[2] If the unknown reading exceeds that of the standard, the sensitivity is reset for the 0.4-microgram standard. If the reading of the unknown is still too high, the 0.1-milliliter ethanol duplicate, saved for this purpose, is suitably diluted and an aliquot is re-assayed with new standards and an equally dilute solvent blank.

Calculations are based upon the following formula:

$$\frac{\text{Unknown Reading} - \text{Solvent Blank}}{\text{Standard Reading} - \text{Acid Blank}} \times \text{Standard } (\mu\text{g.}) \times \text{Dilution} \times \frac{\text{Total Urine Volume (ml.)}}{20 \text{ (ml.)}} = \mu\text{g. of Estriol in specimen.}$$

RESULTS AND DISCUSSION

The sensitivity and accuracy of the method were tested by measuring the recovery of estriol from estriol glucuronide added to urine, by performing duplicate and occasionally quadruplicate assays on urines of different estriol content, and by correlating the results of estriol assays with otherwise measurable variations in the function of endocrine glands known to produce estrogens.

It was established at the beginning of the study that crystalline estriol applied to the alumina column was eluted quantitatively in the final fraction, with a 30 per cent solution of ethanol in benzene. Estrone and estradiol could not be adequately separated from each other, but both were completely eluted in the 2 per cent and 4 per cent ethanolic fractions. A small amount of "estriol glucuronide derived from pregnancy urine" (Sigma Chemical Co.) was available for the performance of recovery experiments. Aqueous solutions of the conjugated material were carried through the routine procedure. It was found that only 46.3–49.0 per cent (average, 47.9 per cent) of the total estrogen content of the solution was eluted from the alumina column in the final estriol fraction; 42.3 per cent was eluted with a 2 per cent solution of ethanol in benzene, and 6.6 per cent of the total estrogen with a 4 per cent solution of ethanol in benzene. Thus although 96.8 per cent of the original estrogen content of the conjugated material was recovered at the end of the experiment, only half of the total represented estriol. To establish this fact conclusively, a 100-μg. portion of the conjugate was hydrolyzed with glucuronidase and the liberated estrogen was chromatographed on six 1-gram alumina columns in the routine manner. The 2 per cent, 4 per cent, and 30 per cent ethanolic fractions

[2] The acid blank should read no higher than 12, and the solvent blank no higher than 16. The addition of 1 drop of 30 per cent hydrogen peroxide was repeatedly found to reduce the fluorescence of both the unknown and the standard to that of the respective blank, hence this additional step was eliminated.

from each column were appropriately pooled and the 3 pools were chromatographed on paper in the solvent system benzene/50% methanol. Estrogens were detected by spraying with a mixture of ferric chloride and potassium ferricyanide (8). The 30%-ethanol pool was found to contain only estriol; the other 2 pools appeared to contain a mixture of estrogens, presumably largely estradiol, but no estriol. It was concluded that the "estriol glucuronide" was a mixture of several estrogens in conjugated form.

Recovery experiments were performed by adding known amounts of the impure conjugate to urine, which was then subjected to the routine procedure. The recovery of estriol was calculated on the basis of the fact that the conjugate contained 47.9 per cent estriol glucuronide. The results of the recovery experiments are shown in Table 1. In the first experiments, acid hydrolysis was employed and the recovery of estriol from the conjugate was found to be reasonably satisfactory. It became apparent later that slight variations in heating the acidified urine in the pressure cooker caused marked changes in both the control values and the recovery of estriol from added conjugate. Prolonged heating caused considerable destruction of estriol. The recovery following enzymatic hydrolysis was equally good or better, in agreement with the findings of Smith and Blackman (7). Previous experiments had demonstrated that the recovery of estriol was not significantly increased under these experimental conditions by increasing the

TABLE 1. ESTRIOL RECOVERY EXPERIMENTS

Urine Specimen	Hydrolysis	Estriol (μg.)			% Recovery
		Found	Added*	Recovered	
1	Acid—15 lbs., 10 mins.	0.249	0.202	0.448	98.5
1	Acid—15 lbs., 10 mins.	0.259	0.202	0.427	83.3
1	Acid—15 lbs., 20 mins.	0.236	0.202	0.364	63.4
1	Acid—15 lbs., 30 mins.	0.199	0.202	0.315	57.4
1	Acid—15 lbs., 60 mins.	0.064	0.202	0.152	48.5
1	Sigma glucuronidase	0.249	0.202	0.451	100.0
2	Sigma glucuronidase	0.061	0.202	0.238	87.6
3	Sigma glucuronidase	0.190	0.202	0.419	113.5
4	Sigma glucuronidase	0.031	0.105	0.130	94.3
4	Sigma glucuronidase	0.032	0.105	0.128	91.4
5	Sigma glucuronidase	0.198	0.105	0.323	109.4
6	Sigma glucuronidase	0.123	0.105	0.218	90.5
6	Sigma glucuronidase	0.123	0.105	0.227	99.2

* Calculated estriol content of estriol glucuronide added. See text.

amount of bacterial glucuronidase added to urine or by prolonging the length of incubation—which findings support and amplify the conclusions drawn by Smith and Blackman (7).

The specificity of the procedure as a measure of urinary estriol was next investigated. These experiments were limited to the assay of estriol in urine containing only small amounts of estrogens, in analysis of which the specificity of the procedure would be especially important. Duplicate aliquots of 8 random urine specimens were subjected to the routine procedure through the chromatographic step. Half of the estriol fractions were treated with dimethyl sulfate in sodium hydroxide solution to form the methyl ether derivatives. The latter were extracted and re-chromatographed on alumina, by the method of Brown (1). The eluted fraction containing estriol methyl ether, as determined by pilot studies in which estriol was similarly treated, was dried and assayed fluorometrically, along with the duplicate which had not been subjected to the methylation step. It is apparent from Table 2 that the results obtained by the 2 methods agreed reasonably well and that the final estriol fraction obtained from urine by the simpler method was sufficiently pure for routine assay purposes.

To test the reproducibility of the procedure, 13 fresh urine specimens of varying estriol content were assayed in duplicate. The results are listed in Table 3. In 2 experiments (M-2 and P-2), the same refrigerated urine was re-assayed forty-eight hours later. In most instances the values agreed within 10 per cent, which is considered satisfactory. The assays of urine containing only minute amounts of estriol appeared to be as reproducible as the assays of urine containing larger quantities.

The significance of the results obtained by this procedure was investi-

TABLE 2. SPECIFICITY OF METHOD: PURITY OF ESTRIOL FRACTION

Specimen	Urinary estriol (μg./24 hrs.) determined	
	by routine method	as methyl-ether derivative*
1	21.9	22.4
2	49.0	57.0
3	50.8	48.5
4	44.4	59.0
5	27.8	34.8
6	26.8	24.4
7	16.2	19.5
8	10.5	8.8

* Duplicate estriol fraction, eluted from alumina column in routine method, subjected to additional methylation step, re-chromatographed, and assayed fluorometrically as estriol methyl ether. See text.

TABLE 3. DUPLICATE ASSAYS OF URINARY ESTRIOL (μG./24 HRS.)

Subject	Assay 1	Assay 2
A	1.2	1.5
B	2.0	2.2
C	3.8	3.9
D	4.5	4.7
E	5.9	6.5
F	5.3	6.7
G	7.3	8.3
H	7.5	8.1
I	8.5	9.0
J	11.1	13.7
K	11.4	13.0
L	12.1	13.2
M-1	14.0	16.2
M-2	15.6	15.6
N	19.1	21.2
O	27.1	28.2
P-1	31.4	32.0
P-2	28.2	30.5
Q	40.0	41.8
R	42.3	42.7

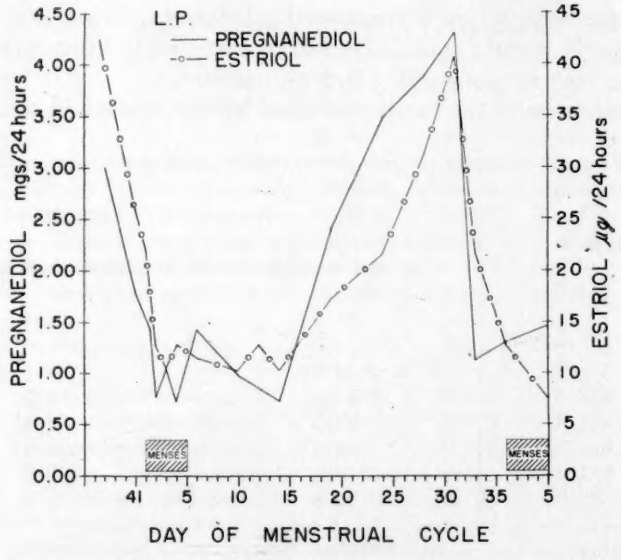

FIG. 1. Excretion of estriol and pregnanediol during menstrual cycle of adult female.

gated by comparing the amount of estriol found in the urine with other clinical and laboratory indices of ovarian function in the adult female. For this study, urine was collected from 3 adult women during the course of a menstrual cycle and analyzed for both estriol and pregnanediol content. Urinary pregnanediol was measured by means of paper chromatography, a method previously described (9). The results of 1 experiment are illustrated in Figure 1. The excretion of estriol was found to parallel the excretion of pregnanediol during 2 consecutive menstrual cycles, rising steeply during the luteal phase and falling abruptly prior to the onset of menstruation. There is close agreement between the absolute amount of estriol excreted daily by this subject as determined by this method, and that reported by Brown (4) in his study of other normal subjects. It would appear that the present method yields results which correlate well with evident cyclic changes in ovarian secretory activity and with the results of other investigators employing more laborious chromatographic techniques for the measurement of urinary estrogens.

The validity of the assay was further tested by studying a group of children before and after the administration of adrenocorticotropin (ACTH) and a larger series of patients with congenital adrenal hyperplasia. The adrenal is known to secrete estrogens (10), and an increased excretion in the urine has been found in cases of adrenal hyperplasia and tumor, using countercurrent distribution (3). Table 4 demonstrates the excessive excretion of estriol, as determined by this method in 13 patients with congenital adrenal hyperplasia prior to treatment, as well as the elevated excretion of 17-ketosteroids and pregnanetriol. The values for estriol agree well with those determined by countercurrent distribution (3). Estriol excretion fell on treatment with cortisone or its analogues, as did the excretion of the other 2 types of steroids, indicating a good correlation between the results obtained by the estriol method and other reliable means of assessing adrenocortical overactivity in this disease.

In some patients the determination of estriol excretion seemed to serve as a more sensitive index of abnormal adrenal function than the measurement of 17-ketosteroid excretion (Table 4, Subject F.Sm.). The method could clearly detect the rapid resumption of adrenocortical overactivity following cessation of steroid therapy, as well as the suppression of the gland when treatment was resumed (Subject M.St.). It permitted the detection of increased estrogen secretion by the normal adrenal in response to the administration of ACTH, as shown in Table 5. In the case of Addison's disease (Subject R.C., Table 5), the estriol method supplied important information which helped to confirm the clinical diagnosis of adrenal insufficiency.

TABLE 4. RELATIONSHIP OF ESTRIOL TO 17-KETOSTEROID AND PREGNANETRIOL EXCRETION IN CONGENITAL ADRENAL HYPERPLASIA

Subject	Sex	Age (yrs.)	Treatment	Estriol (μg./4 hrs.)	17-KS (mg./24 hrs.)	Pregnanetriol (mg./24 hrs.)
J.So.*	M	$\frac{1}{12}$	0	37.9	14.9	9.8
G.Kr.*	M	$\frac{2}{12}$	0	31.0	2.6	2.9
G.Ac.	F	$\frac{3}{12}$	0	6.0	2.5	1.8
C.Ho.*	F	$1\frac{4}{12}$	Desoxycorticosterone	30.7	5.1	15.3
L.Bl.	F	$1\frac{6}{12}$	0	17.3	3.2	8.4
A.Th.	F	$1\frac{7}{12}$	0	27.7	4.1	4.6
			Hydrocortisone	10.8	2.7	0.8
R.Be.	F	2	0	113.0	20.9	16.6
			Cortisone	1.8	7.5	0.1
F.Sm.	M	6	0	39.9	5.4	5.4
			Medrol	0.0	4.0	1.7
R.Co.	M	$6\frac{3}{12}$	0	10.5	10.0	12.5
E.Fl.	M	7	0	69.0	13.8	56.0
			Cortisone-? dose†	11.8	21.1	11.6
B.Br.	F	15	0	124.8	38.1	27.1
J.Bs.	F	$15\frac{6}{12}$	0	60.8	33.5	30.2
M.St.‡	F	21	Cortisone	19.1	17.5	1.0
			No cortisone 24 hrs.	23.4	15.5	3.6
			No cortisone 48 hrs.	65.7	28.1	3.5
			Medrol, 1 wk.	3.8	9.5	0.6
			Medrol, 2 wks.	3.9	5.6	1.1
Normal		<1 yr.		0.0–1.0		
		1–5		1.0–3.0		
		6–10		2.0–6.0		

* Salt-losing form of disease.
† Inadequate treatment.
‡ Hypertensive form of disease.

TABLE 5. EFFECT OF ACTH* ON ESTRIOL EXCRETION

Subject	Age (yrs.)	Diagnosis	Date	Treatment	Estriol (μg./24 hrs.)	17-KS (mg./24 hrs.)
J.B.	$\frac{3}{12}$	Congen. adrenal hyperplasia†	July 5	0	28.2	3.5
			July 6	ACTH	39.3	9.1
M.P.	$5\frac{6}{12}$	Normal	June 23	0	3.1	0.2
			June 24	ACTH	6.8	1.6
R.N.	6	Hypoglycemia	Feb. 11	0	2.9	1.3
			Feb. 14	ACTH, 3 days	6.9	2.2
			Feb. 21	No ACTH for 7 days	4.4	0.7
			Feb. 24	ACTH, 3 days	9.7	1.8
J.C.	$13\frac{6}{12}$	Adolescent gynecomastia	June 15	0	7.0	4.0
			June 16	ACTH	13.9	7.1
			June 17	ACTH	13.1	10.0
R.C.	13	Addison's disease	Oct. 6	0	0.0	1.8
			Oct. 9	ACTH, 3 days	0.0	1.6
G.H.	23	Amenorrhea	Feb. 12	0	15.6	—
			Feb. 15	ACTH, 3 days	31.7	—

* A dose of 15 mg. of ACTHAR gel per sq. M. was injected intramuscularly daily for 1–3 days.

† Salt-losing form.

The method has been applied to a study of the excretion of estrogens during infancy, childhood and adolescence. (The results of these studies will be reported elsewhere.) Estriol excretion was found to rise with advancing age in normal children and to vary little between males and females of the same age until adolescence is reached.

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THE URINARY EXCRETION OF TETRAHYDROCORTISOL, 3α-ALLOTETRAHYDROCORTISOL AND TETRAHYDROCORTISONE IN YOUNG AND ELDERLY MEN AND WOMEN*

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ABSTRACT

Tetrahydrocortisol, 3α-allotetrahydrocortisol and tetrahydrocortisone are regularly occurring metabolites in the urine of young and elderly men and women. The rate of excretion of these steroids, expressed as a function of creatinine excretion, is not altered by age or sex. The average proportions of each of these metabolites to their total excretion is approximately 30 per cent for tetrahydrocortisol, 20 per cent for 3α-allotetrahydrocortisol and 50 per cent for tetrahydrocortisone.

IN PAPERS (1, 2) published previously in this journal the authors described a method for the quantitative determination of urinary tetrahydrocortisol (THF), 3α-allotetrahydrocortisol (ATHF) and tetrahydrocortisone (THE), and also reported data obtained by this method in a study of young normal and schizophrenic men. The diurnal and 24-hour excretion of these steroid metabolites was presented in terms of Porter-Silber (PS) chromogen after the ethyl-acetate extract of urine incubated with β-glucuronidase had been separated by paper chromatography into the 3 steroid components. Their identity was established by determinations of sulfuric-acid chromogen spectra and by crystallization and identification by means of infrared analysis. All 3 metabolites appeared not only to occur regularly in normal and schizophrenic men but to be excreted by both groups of subjects with a certain amount of constancy as regards both the absolute output and the proportion of each steroid to the total output. Accordingly, in order to determine whether the age or sex of the subject had any effect on the excretion of these 3 prominent urinary metabolites, samples of urine were collected from 10 young men, 10 young women, 10 elderly men, and 10 elderly women, and quantitative estimations were made of the THF, ATHF and THE present in each.

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METHODS

The young male and female subjects, consisting of laboratory personnel, contributed 24-hour urine samples, of which 12-hour aliquots were extracted for subsequent determinations. The samples were not collected from the female subjects at any particular phase of the menstrual cycle. The elderly subjects, with 2 exceptions, were residing in homes for the aged, were in reasonably good health and were receiving no medications. Complete 24-hour urine samples were utilized in the case of the elderly women, and either 12-hour aliquots or 24-hour samples in the case of the elderly men.

RESULTS

The steroid metabolites, both as the total amount in the initial extract and as THF, ATHF and THE, were measured as PS-chromogen with THE as the reference standard. The PS reaction was carried out for one hour in a water bath at 60°C. Under these circumstances, concentrations of THF and ATHF equivalent to those of THE had optical densities (O.D.) which were 50 and 48 per cent respectively of those for THE. (When the PS reaction was carried out overnight at room temperature, equivalent concentrations of THF and ATHF had O.D.'s 75 to 80 per cent of those for THE). The results are therefore expressed in mg. per twenty-four hours, first in terms of THE standard, and then with THF and ATHF values calculated using each steroid as its own reference standard. In Table 1 are listed the mean urinary excretion values in terms of THE standard, 1) for the total extract prior to paper chromatography, 2) for THF, ATHF and THE after the initial separation in the benzene-formamide system, and 3) for THF, ATHF and THE after the final separation in the chloroform-formamide system. The mean percentage recovery of the total PS as THF, ATHF and THE is indicated in the last column.

A comparison of the values obtained with the 2 chromatography systems indicates the general reproducibility of results with paper chromatography. In addition, the 3 metabolites account for approximately 75 per cent of the 17-hydroxycorticosteroids present in the original extracts. This value compares with a mean recovery of 74–75 per cent previously obtained (1, 2).

The young men had outputs of total PS, THF and THE similar in range to those found for young men in previous studies but significantly higher than those for young and elderly women and elderly men. The significant differences as T values are presented in Table 2. Among the latter 3 groups the excretion of these metabolites was not quantitatively different. The excretion of ATHF was not significantly different between any of the 4 groups.

In Table 3 the mean PS values are presented for THF and ATHF after

TABLE 1. MEAN EXCRETION VALUES OF TOTAL PS, THF, ATHF AND THE (MG./24 HRS.)*
FOR YOUNG AND ELDERLY MEN AND WOMEN

Subjects	Median age (yrs.)	Creatinine (Gm./24 hrs.)	Total extract	Benzene-formamide			Chloroform-formamide				% THF, ATHF, THE of Total PS
				THF	ATHF+THE	THF, ATHF +THE	THF	ATHF	THE	THF, ATHF +THE	
Young men	30 18–35‡	1.6±0.07† 1.4–2.1‡	6.0±0.34 4.5–7.9	1.0±0.09 0.6–1.4	4.0±0.34 1.9–5.4	5.0±0.38 2.5–6.4	1.0±0.09 0.6–1.4	0.5±0.10 0.2–1.2	3.4±0.34 1.8–5.3	4.9±0.37 2.6–6.4	82.3±4.2
Young women	25 22–38	1.3±0.07 0.8–1.6	4.4±0.35 2.6–6.0	0.5±0.09 0.2–1.1	2.7±0.34 1.9–5.7	3.2±0.34 2.1–6.0	0.5±0.07 0.3–0.9	0.3±0.06 0.1–0.7	2.2±0.16 1.7–3.5	3.0±0.20 2.2–4.1	70.0±4.3
Older men	80 74–87	1.1±0.10 0.6–1.5	3.9±0.54 1.8–6.8	0.6±0.11 0.1–1.2	2.2±0.35 0.7–4.2	2.8±0.43 0.8–5.4	0.6±0.09 0.2–1.1	0.5±0.07 0.1–0.7	1.9±0.25 0.7–3.0	3.0±0.38 1.0–4.6	76.0±5.9
Older women	80 64–80	1.0±0.07 0.8–1.3	3.7±0.20 2.8–4.7	0.6±0.08 0.3–0.9	2.0±0.28 1.0–3.1	2.6±0.34 1.3–4.0	0.6±0.06 0.3–0.8	0.4±0.08 0.1–0.7	1.7±0.22 0.9–2.5	2.7±0.27 1.3–3.6	70.6±5.5

* Expressed in THE equivalents.
† Standard error.
‡ Range.

TABLE 2. SIGNIFICANT DIFFERENCES (T VALUES), IN TOTAL PS, THF AND THE OUTPUT (MG./24 HRS.) BETWEEN YOUNG MEN AND YOUNG AND OLDER WOMEN AND OLDER MEN*

	Benzene-formamide				Chloroform-formamide			
Young men *vs.*	Total PS	THF	ATHF + THE	THF, ATHF, THE	THF	ATHF	THE	THF, ATHF, THE
Young women	3.33	4.14	2.60	3.51	4.55	N.S.†	3.59	4.60
Older men	3.36	2.90	3.62	3.73	3.23	N.S.	3.70	3.83
Older women	5.89	3.93	4.52	4.80	4.06	N.S.	5.24	4.99

* --- 2% level of confidence.
— 1% level of confidence.
═ 0.1% level of confidence.
† Not significant.

calculation of all THF values in terms of THF standard and all ATHF values in terms of ATHF standard in order to approximate more nearly their actual urinary levels. Thus THF and ATHF constitute roughly half of the total output of THF, ATHF and THE combined.

In view of the age and sex differences in such factors as body weight and renal function, each subject's PS values were calculated in terms of mg.

TABLE 3. MEAN PS EXCRETION VALUES (MG./24 HRS.) FOR THF EXPRESSED IN THF EQUIVALENTS, ATHF EXPRESSED IN ATHF EQUIVALENTS, AND THE EXPRESSED IN THE EQUIVALENTS

Subjects	Chloroform-formamide system			
	THF	ATHF	THE	THF, ATHF +THE
Young men	2.0±0.17 1.4–2.9	1.1±0.20 .4–2.4	3.4±0.34 1.8–5.3	6.5±0.46 3.4–8.0
Young women	0.9±0.13 .5–1.8	0.7±0.11 .4–1.4	2.2±0.16 1.7–3.5	3.8±0.28 2.6–4.8
Older men	1.1±0.18 .3–2.2	0.9±0.14 .3–1.5	1.9±0.25 0.7–3.0	3.9±0.53 1.2–6.3
Older women	1.1±0.11 .5–1.6	0.8±0.16 .2–1.5	1.7±0.22 0.9–2.5	3.6±0.36 1.7–4.9

per Gm. of creatinine output. The mean and range of values are presented in Table 4. This correction for muscle mass and so forth eliminated any significant differences due either to age or sex in the quantitative excretion of total PS, THF, ATHF and THE—with 1 exception. The excretion of THF (0.7 mg.) for the young women was significantly smaller ($T=3.46$; 0.1 per cent level of confidence (L.C.)) than the output of 1.2 mg. for the young men, 1.3 mg. for the older women ($T=2.95$, 1 per cent L.C.), and 1.0 mg. for the older men ($T=2.29$, 5 per cent L.C.). The low value of 0.7 mg. of THF for the young women contributed sufficiently to the sum of THF, ATF and THE to make their total excretion (3.0 mg.) significantly lower than that (4.0 mg.) for the young men. For all subjects, the mean excretion values for THF in terms of THF standard, for ATHF in terms of ATHF standard, and for THE in terms of THE standard were respectively 1.1, 0.7 and 1.8 mg. per Gm. of creatinine $\times 100$.

The mean ratios of excretion of each steroid to the sum of the 3 steroids for young and elderly subjects are given for both chromatogram systems in Table 5. For all subjects, approximately 30 per cent of the total THF, ATHF and THE output was accounted for as THF; 20 per cent as ATHF; and 50 per cent as THE. In young female subjects the ratio for THF excretion was 24 per cent, which is significantly lower than the 31 per cent for young men ($T=2.26$, 5 per cent L.C.) and 32 per cent for older women ($T=2.81$, 2 per cent L.C.). The THE ratio of 59 per cent for young women was significantly higher than the ratio of 48 per cent for older men ($T=2.32$, 5 per cent L.C.).

The identity of ATHF was further established after paper chromatography and determination of sulfuric-acid spectra by crystallization of the steroid and its identification by infrared analysis (I.R.A.). Crystalline material was obtained and identified for each of 9 young men. ATHF was isolated from paper eluates for each of 2 young women, and from a pool of eluates for 4 female subjects who had insufficient ATHF for individual isolations. The ATHF from 2 subjects was completely used in preliminary estimations. No crystalline material was obtained from a pool of ATHF from the 2 remaining female subjects; however, further chromatography and sulfuric-acid chromogen determinations indicated that the small amount of material present was very likely ATHF.

ATHF from paper chromatogram eluates for 8 older men were pooled, and crystalline ATHF isolated and identified. Crystalline ATHF was also obtained from the eluates for each of the 2 remaining older men. ATHF was isolated from each of 6 older women's samples and from a pool of ATHF eluates from the remaining 4 subjects.

As shown in Table 6, 2 young men, 1 young woman, 2 elderly men and 4 elderly women seemed to have relatively higher absolute and proportional

TABLE 4. THE MEAN PS EXCRETION VALUES IN THE CHLOROFORM-FORMAMIDE SYSTEM FOR THF, ATHF AND THE EXPRESSED AS MG. PER GM. OF CREATININE OUTPUT

Subjects	THE as reference standard					Each steroid as its own reference standard			
	Total extract	THF	ATHF	THE	THF, ATHF +THE	THF	ATHF	THE	THF, ATHF +THE
Young men	3.7 ±0.22 2.8–5.0	0.6 ±0.04 0.3–0.8	0.3 ±0.06 0.1–0.7	2.1 ±0.24 1.1–3.4	3.0 ±0.25 1.5–4.1	1.2 ±0.09 0.7–1.6	0.7 ±1.12 0.3–1.4	2.1 ±0.24 1.1–3.4	4.0 ±0.29 2.1–5.0
Young women	3.4 ±0.36 2.2–5.9	0.4 ±0.06 0.2–0.7	0.3 ±0.05 0.1–0.5	1.7 ±0.13 1.3–2.6	2.4 ±0.17 1.6–3.4	0.7 ±0.10 0.4–1.1	0.6 ±0.11 0.1–1.1	1.7 ±0.13 1.3–2.6	3.0 ±0.26 1.8–4.3
Older men	3.5 ±0.35 2.3–5.2	0.5 ±0.06 0.2–0.8	0.4 ±0.05 0.1–0.6	1.7 ±0.15 1.0–2.5	2.6 ±0.26 1.3–3.8	1.0 ±0.14 0.3–1.6	0.8 ±0.12 0.2–1.3	1.7 ±0.15 1.0–2.5	3.5 ±0.36 1.6–5.2
Older women	4.0 ±0.23 2.3–4.8	0.6 ±0.08 0.3–1.1	0.4 ±0.12 0.1–1.2	1.8 ±0.30 1.0–3.0	2.8 ±0.32 1.4–4.5	1.3 ±0.16 0.5–1.6	0.9 ±0.25 0.2–2.5	1.8 ±0.30 1.0–3.0	4.0 ±0.49 1.9–6.2
All subjects	3.6 ±0.15 2.2–5.9	0.5 ±0.04 0.2–1.1	0.4 ±0.04 0.1–1.2	1.8 ±0.10 1.0–3.4	2.7 ±0.13 1.3–4.5	1.1 ±0.07 0.3–1.6	0.7 ±0.08 0.1–2.5	1.8 ±0.10 1.0–3.4	3.6 ±0.20 1.6–6.2

TABLE 5. THE MEAN RATIO OF EXCRETION OF THF, ATHF AND THE TO THEIR SUM TOTAL OUTPUT

Subjects	Benzene-formamide*		Chloroform-formamide*			Chloroform-formamide†		
	THF	ATF +THE	THF	ATHF	THE	THF	ATHF	THE
Young men	21±1.5	79±1.5	21±1.8	11±1.9	68±2.9	31±2.3	16±2.5	53±3.4
Young women	15±2.3	85±2.3	15±1.7	10±1.5	75±2.5	24±2.1	17±2.1	59±3.0
Older men	22±2.9	78±2.9	21±2.0	15±1.3	64±2.2	29±2.4	23±1.9	48±2.4
Older women	22±1.6	78±1.6	22±1.7	14±3.0	64±3.7	32±1.8	21±3.6	47±3.9
All subjects						29±1.2	19±1.3	52±1.7

* Each metabolite expressed in THE equivalents.
† Each metabolite expressed in own equivalents.

outputs of ATHF than the other subjects. The preponderance of older women among these subjects may have been the result of selection of 10 adequate urine collections from among 24 received. No such collection difficulties were encountered with the other 3 groups of subjects. When comparisons are made with the means and ranges of values listed in Table 2 for the respective group of subjects, it is obvious that the foregoing subjects contributed the ATHF values that were at the upper limit of the respective ranges. The values of THF and the total of THF, ATHF and THE for these subjects tended to be at the level of, or greater than the means for, comparable groups. The mean value of 31 per cent for the ratio of ATHF to total THF, ATHF and THE output for these 9 subjects was significantly higher ($T=5.91$, 0.1 per cent L.C.) than that of 19 per cent for all subjects, and the ratio of 41 per cent for THE was significantly lower ($T=3.56$, 0.1 per cent L.C.) than that of 52 per cent for all subjects. The ratios of THF (28 and 29 per cent, respectively) were the same for both groups.

In order partially to confirm the validity of these high ATHF values, the crude crystalline ATHF isolated from the paper eluates for these subjects was weighed on a microbalance and the recovery by weight compared to the amount indicated as being present by PS determination on an aliquot of the eluate taken prior to crystallization. The results are presented in Table 6. The minimum recovery by weight of 60 per cent, and the mean recovery of 70 per cent, suggest that the observed high PS values were reasonably valid.

TABLE 6. PS VALUES, RATIOS AND RECOVERY BY WEIGHT OF ATHF IN SUBJECTS WHO EXCRETED ATHF IN ABOVE-AVERAGE AMOUNTS

Subject	PS (mg./24 hrs.)*				Ratio to total			PS (mg.)* ATHF for cryst.	Wt. (mg.) of cryst. ATHF	% Recovery	Melting point (°C.)
	THF	ATHF	THE	Total	THF	ATHF	THE				
Young man P	2.6	2.4	3.0	8.0	32	30	38	.42	.31	74	237.5–245.0†
Young man G	1.8	1.8	2.5	6.1	29	30	41	.36	.21	58	239.0–250.0†
Young woman R	1.1	1.4	2.3	4.8	23	29	48	.32	.28	88	246.5–260.0§
Older man R	1.3	1.3	2.4	5.0	26	26	48	.26	.19	73	245.0–262.0§
Older man J	1.5	1.4	2.4	5.3	28	26	46	.46	.26	57	234.0–241.0†
Older woman R	1.3	1.5	0.9	3.7	35	40	25	.40	.26	65	236.0–240.0†
Older woman D	1.2	1.2	1.9	4.3	28	35	37	.44	.33	75	232.0–240.0†
Older woman H	1.1	1.5	2.4	5.0	22	30	48	.20	.12	60	238.0–247.0†
Older woman S	1.4	1.2	1.0	3.6	29	33	38	.32	.20	63	233.0–241.5†
Mean total	1.5	1.4	2.1	5.0	28.0±1.4	31.0±1.5	41.0±2.5			68.1	

* Each metabolite expressed in own equivalents.

† Melting point of an authentic sample of ATHF in crushed form, 240.0°–246.5° C.

§ Melting point of an authentic sample of ATHF as intact crystals, 262.0°–265.0° C.

DISCUSSION

In a previous study of urinary steroid excretion patterns in 23 young and 14 elderly persons of both sexes (3), THF and THE—as characterized by running rates and color reactions on paper chromatograms, sulfuric-acid chromogen spectra on eluates therefrom, and crystallization from pools of eluates—were found in the urine extracts in all cases. However, no quantitative estimations of these steroids were made.

Subsequently quantitative determinations of THF and a combination of ATHF and THE were carried out on two-hour aliquots of samples from a series of young Air Force personnel and schizophrenic men (1). Not only did these metabolites occur in a quantitatively regular way but, as might be expected, there was a diurnal rhythm in their excretion. The results on 12-hour aliquots of 24-hour urine samples from an additional series of normal and schizophrenic men indicated that in these subjects ATHF was a regularly occurring metabolite which formed a rather constant proportion of the total excretion of THF, ATHF and THE (2).

The excretory levels of THF, ATHF and THE (THE reference standard) in terms of milligrams of PS per twenty-four hours for the young men in the present study are similar to those obtained in the 2 studies cited and in those reported by Bush and Willoughby (4). In the young and older women and older men the 24-hour mean quantitative output of the 3 metabolites was significantly less than in young men. However, when their urinary excretion is expressed as a function of creatinine excretion, the differences in the quantitative excretion of these metabolites between male and female and young and elderly disappear, with 1 exception.

This exception was provided by the young women, in whom, at least in this particular sampling, the output of THF was significantly lower than in the other 3 groups of subjects. Further determinations are required in order to substantiate whether these low values, seemingly characteristic, are the result of 1) incomplete solvent extraction of this polar steroid, 2) loss prior to, or during paper chromatography in the benzene-formamide system due to some particular lability, or 3) a relationship to the time of collection during the menstrual cycle. Maengwyn-Davies and Weiner (5), for example, found that the excretion of 17-hydroxycorticosteroids was significantly higher in the second and third week as compared to the first and fourth week of the cycle.

Irrespective of the quantitative differences in the absolute output of THF, ATHF and THE in the 4 categories of subjects, the average proportion of each of these metabolites to their total output was similar for all groups, with the exception of the young women. There was a tendency for the young subjects to exhibit a non-significant but smaller proportion of

ATHF than the older subjects. However, the similarity of the mean ratios of PS outputs of the 3 metabolites in terms of creatinine suggest that, in general, the metabolism of the precursor or precursors of THF, ATHF and THE is not altered particularly by the age or sex of the subject.

Nevertheless, certain subjects (Table 6), at least on the day of the urine collection, metabolized these steroids in a somewhat different manner—characterized by absolute and proportional increases in the output of ATHF. This increase appeared to be at the expense of the output of THE, which declined proportionately. All these subjects had total THF, ATHF and THE outputs as high or higher than the average output for their respective groups. This fact suggests the possibility of a quantitative shift in favor of ATHF when the level of adrenocortical steroid excretion is high Thus, stress or administration of ACTH might enhance the excretion of this metabolite. Further investigations are necessary to elucidate the relationship between the urinary excretions of THF, ATHF and THE.

In regard to the question raised by Bush and Willoughby (4) as to the probable steroid precursor of ATHF, the isolation of labeled ATHF after administration of 4-C^{14} labeled cortisol together with non-radioactive cortisol indicates that the latter, as suggested, may well be the parent steroid. This is demonstrated by the following study. An aliquot (85 per cent) of a 24-hour urine sample collected immediately after intravenous administration of 1.2 μc. of cortisol-4-C^{14} and 100 mg. of non-radioactive cortisol to a female patient was kindly provided by Dr. Lewis Engel, Massachusetts General Hospital. After hydrolysis with bacterial glucuronidase, continuous extraction was carried out with methylene chloride. The extract was submitted to paper chromatography for separation of the reduced cortisol metabolites. A crystalline material was obtained, the infrared spectrum of which was identical with that of an authentic sample of ATHF. Two subsequent recrystallizations were made. The melting points and radioactivity determined on duplicate samples from each crystallization were as follows: 1) M.P. 238.5–240.5° C.; radioactivity, 11,345–11,520 cts./min./mg. 2) M.P. 246.0–248.5° C.; radioactivity, 9,606–9,879 cts./min./mg. 3) M.P. 243.0–246.5° C.; radioactivity, 10,100–9,733 cts./min./mg.

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THE INFLUENCE OF TRANQUILIZING DRUGS ON RESULTS OF THYROID FUNCTION STUDIES

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ABSTRACT

A study was undertaken to ascertain whether the ingestion of a tranquilizing drug alters the results of thyroid function studies. In 31 euthyroid and 12 hyperthyroid patients, the thyroidal 24-hour radioiodine uptake and the serum cholesterol and protein-bound iodine levels were determined. These tests were repeated after ten to twenty-one days of therapy with promazine, meprobamate, hydroxyzine or reserpine. Statistical evaluation failed to reveal any significant difference in the results of thyroid function tests due to administration of these tranquilizing drugs.

INTRODUCTION

TRANQUILIZING drugs may alter signs and symptoms in hyperthyroid or in tense euthyroid patients, thereby making the clinical diagnosis more difficult. It is important to determine whether these drugs also alter the laboratory findings, because frequently patients referred for thyroid function studies are taking ataractics. In an effort to evaluate the effects of these medications, groups of clinically hyperthyroid and euthyroid patients were studied before and during therapy with tranquilizers.

CLINICAL MATERIAL AND METHODS

The 24-hour thyroidal I^{131} uptake, and the levels of serum cholesterol and protein-bound iodine were determined in 31 euthyroid and 12 hyperthyroid patients before medication and again during administration of one of the tranquilizing drugs. Most of the euthyroid patients were healthy young adults with no history of thyroid disease, and careful physical examination did not reveal any symptoms or signs suggesting thyroid dysfunction. The group with hyperthyroidism had typical clinical features of thyrotoxicosis at the time of the study, and all subsequently responded to medical, surgical or radioactive iodine (I^{131}) therapy.

The 4 drugs used belong to separate groups of ataractics, supposedly having different sites of action. They were administered orally, in 3 or 4 doses daily for at least ten days before thyroid function studies were repeated. The drugs were: reserpine (a Rauwolfia

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alkaloid), 0.75 mg. per day; promazine (Sparine),[1] 200 mg. per day; meprobamate (Equanil),[1] 1600 mg. per day; and hydroxyzine (Atarax),[1] 200–300 mg. per day.

The patients received no other medications during the period of study. Each patient was questioned with reference to side-effects or a change in symptomatology, and examined for objective effects.

The thyroidal I^{131} uptake was routinely determined approximately twenty-four hours following the ingestion of 5 to 10 microcuries of carrier-free sodium radioiodide. The euthyroid range in our laboratory is between 15 per cent and 40 per cent of the administered dose. The concentration of serum protein-bound iodine (PBI) was measured by the method of Barker (1), and that of serum cholesterol by the method of Zak (2). Our normal range for serum PBI is 3.7 to 7.5 micrograms per 100 ml., and for serum cholesterol, 150 to 300 mg. per 100 ml. Any values outside the foregoing ranges were considered abnormal even though they may have been within the general borderline range for these tests. By the method of Brownell (3), a concentration of less than 5 per cent of the radioactive iodine in the gland per hour represents the normal rate. Failure to suppress the thyroidal uptake during administration of 3 gr. of desiccated thyroid daily for one month is considered characteristic of thyrotoxicosis.

RESULTS

In Table 1 the laboratory findings in all the euthyroid patients are recorded. In Table 2 are listed the findings in all the hyperthyroid patients.

Atarax study

Eight euthyroid patients had normal thyroidal I^{131} uptakes and normal levels of serum cholesterol and PBI both before and during Atarax therapy, with the exception of Patient No. 4 who had a low I^{131} uptake before, but a normal uptake with Atarax. There appeared to be no significant change in the parameters of thyroid function during ingestion of Atarax in these euthyroid patients. The statistical analysis of all groups is summarized in a succeeding paragraph.

In 4 clinically hyperthyroid patients there was no symptomatic or objective improvement while taking the medication. Patient No. 32 had a normal serum PBI level before and during therapy. He had signs and symptoms of diffuse toxic goiter associated with a small gland and an abnormally high I^{131} uptake. Therapy with radioactive iodine was followed by complete disappearance of thyrotoxicosis. With no medication, Patient No. 33 had a normal thyroidal I^{131} uptake and a normal serum PBI level. However, his thyroid concentrated I^{131} at an abnormally rapid rate of 9 per cent per hour, and he was clinically toxic. During administration of Atarax, both the I^{131} uptake and the PBI level rose to hyperthyroid values. Therapy with radioactive iodine was followed by definite clinical improve-

[1] Sparine and Equanil are marketed by Wyeth Laboratories, and Atarax by the Roerig Division of Chas. Pfizer & Company, Inc. We are indebted to both companies for ample supplies of these drugs.

TABLE 1. EFFECT OF TRANQUILIZING DRUGS ON THYROID FUNCTION STUDIES IN EUTHYROID PATIENTS

Drug	Case No.	Thyroidal I^{131} 24-hr. uptake (%)		Serum cholesterol (mg./100 ml.)		Serum PBI (μg./100 ml.)	
		Before	During	Before	During	Before	During
Atarax	1	39	36	188	179	4.4	4.8
	2	31	23	253	205	3.8	4.6
	3	28	27	265	230	4.4	4.6
	4	13	22	196	198	4.8	4.0
	5	24	21	256	230	4.0	4.3
	6	16	18	155	165	4.1	4.2
	7	16	20	220	186	4.2	3.4
	8	18	18	188	160	4.9	5.2
Equanil	9	26	22	274	234	6.1	5.1
	10	32	21	161	139	4.3	4.6
	11	25	23	154	145	4.4	4.3
	12	28	29	164	197	5.5	4.9
	13	26	24	146	204	6.1	6.3
	14	22	23	280	300	4.4	6.0
	15	30	38	146	179	5.8	5.7
Sparine	16	23	28	234	206	5.6	6.5
	17	16	23	172	219	4.3	6.5
	18	13	19	234	164	4.8	3.8
	19	25	24	277	175	6.2	4.0
	20	25	23	229	182	6.3	4.1
	21	18	20	175	175	3.7	6.1
Reserpine	22	18	24	233	299	6.0	7.8
	23	21	27	132	132	6.6	8.4
	24	18	32	299	267	6.0	4.3
	25	15	20	308	296	6.3	4.2
	26	14	13	212	219	4.3	4.6
	27	18	24	158	268	7.3	5.2
	28	23	20	277	285	7.1	5.2
	29	14	15	208	208	8.7	5.8
	30	23	17	186	159	6.4	5.4
	31	12	10	151	168	7.4 4.7	4.6
Mean		21.6	22.7	211	206	5.4	5.1

TABLE 2. EFFECT OF TRANQUILIZING DRUGS ON THYROID FUNCTION STUDIES IN HYPERTHYROID PATIENTS

Drug	Case	Thyroidal I^{131} 24-hr. uptake (%)		Serum cholesterol (mg./100 ml.)		Serum PBI (μg./100 ml.)		Remarks
		Before	During	Before	During	Before	During	
Atarax	32	77	67	159	146	7.4	6.6	Clinically toxic; improved by I^{131} Rx
	33	33	56	139	153	6.3	8.0	Clinically toxic; uptake rate, 9% per hr.
	34	70	64	135	204	12.8	14.0	
	35	62	64	163		22.0	18.4	
Equanil	36	48	46	204	164	6.8	30.0	Surgery for diffuse toxic goiter
	37	54	53	153	172	16.0	17.2	
	38	72	57	116	171	12.8	13.6	Surgery for toxic nodular goiter
	39	83	69	131	131	30.0	30.0	
	40	42	50		179	12.3	9.3	
	41	42	50	164	179	9.5	8.3	
Sparine	42	37	34	146	182	4.5	13.8	No suppression of uptake by thyroid Rx
	43	66	77	117	139	30.0	30.0	
	Mean	57.2	57.3	146	164	14.2	16.6	

ment. In the other 2 hyperthyroid patients, there was no significant change in the results of these laboratory tests.

Equanil study

Seven clinically euthyroid patients had thyroidal I^{131} uptakes and serum levels of cholesterol and PBI within the normal range before and during Equanil administration. In 1 other patient, not included in the study, chills, fever, headache and a petechial rash associated with generalized pruritus developed after the second dose of Equanil. The medication was discontinued and the symptoms gradually disappeared during the next seven days. No previous history of allergy, drug reaction, or ingestion of Equanil could be elicited in this case. The 7 patients in this series noted no undue symptoms except for mild somnolence during the first forty-eight hours.

Six hyperthyroid patients experienced little subjective or objective improvement. With 1 exception, the laboratory findings were essentially unchanged. In Patient No. 36, the concentration of serum PBI was initially in the normal range. During the next three weeks, while taking Equanil, her symptoms became more severe and the PBI concentration rose to a very high level. She responded well to subtotal thyroidectomy. The histologic diagnosis was diffuse toxic goiter.

Sparine study

Of 8 euthyroid patients, 2 spontaneously stopped taking Sparine be-

cause of somnolence or dizziness; their data are not included in the tables. The other 6 experienced no symptoms and there was no apparent change in the results of thyroid function studies.

Of the 2 hyperthyroid subjects, Patient No. 42 had a high-normal thyroidal I^{131} uptake, which failed to decrease during a thyroid suppression test. Initially, the serum PBI level was normal, but ten days later, during administration of Sparine, the PBI level was abnormally high. This man manifested the clinical picture of diffuse toxic goiter. His symptoms were later improved by thyroidectomy, and the diagnosis was confirmed at operation. In Patient No. 43 the laboratory findings were within the hyperthyroid range and did not change during administration of Sparine.

Reserpine study

Because reserpine has already been evaluated in patients with diffuse toxic goiter, we limited our study to 11 euthyroid patients. One discontinued the medication because of nausea. The others had minimal side-effects and completed the study. Three patients (Nos. 26, 29 and 31) had borderline low I^{131} uptakes before and during administration of reserpine. Three patients (Nos. 22, 23 and 29) had elevated serum PBI levels either before or during therapy with reserpine. Otherwise the laboratory findings appeared to be unaltered by reserpine.

Only the data on those patients completing the study were included in the tables and in the statistical analysis. Values in the borderline range were considered as abnormal for statistical purposes. The study included a total of 127 individual determinations during the period without medication, and 127 repeated while the patients were taking tranquilizing drugs. In 107 determinations before, and 110 during therapy, the values were within the range corresponding with that patient's clinical state of thyroid function. The serum PBI level and thyroidal I^{131} uptake reflected the state of thyroid function better than did the serum cholesterol level, both before and during therapy. Application of the *t* test to evaluate the significance of the differences observed between paired experiments, showed differences not in excess of that expected by chance alone.

DISCUSSION

Reserpine has been shown to relieve the symptoms and signs of thyrotoxicosis without influencing the thyroidal I^{131} uptake or the serum PBI level (4). It is felt that reserpine affects the clinical manifestations but not the basic disorder in diffuse toxic goiter. The tranquilizing drugs apparently produce their effects by acting on the subcortical areas, especially the reticular system and the hypothalamus (5, 6). However, the results of studies in rats suggest that Rauwolfia alkaloids may inhibit the synthesis

of thyroglobulin (7) and may thereby affect the thyroid function directly in euthyroid subjects. In our series an attempt was made to evaluate the influence of tranquilizing drugs in both hyperthyroid and euthyroid patients. We tested 1 drug from each of 4 different groups of ataractics, supposedly acting at 4 different sites. Presumably, other ataractic drugs in these groups would have similar effects on thyroid function. All of the medications produced mild side-effects such as somnolence, dizziness or nausea. In 1 patient, however, chills, fever and a petechial rash developed after the second dose of Equanil.

Thyroidal 24-hour I^{131} uptake and serum PBI and cholesterol levels in all groups satisfactorily reflected the clinical status of the patient, indicating that these determinations are reliable for measurement of thyroid function during administration of tranquilizing drugs. Statistical analysis failed to reveal any significant difference between results obtained before and during therapy.

The findings in this series of 31 euthyroid and 12 hyperthyroid patients indicate that the administration of promazine, meprobamate, hydroxyzine or reserpine in the usual therapeutic doses for a period of ten to twenty-one days does not alter the thyroidal I^{131} uptake or the serum levels of cholesterol and PBI in euthyroid or hyperthyroid patients.

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HYPERTROPHIC OSTEOARTHROPATHY AND PRETIBIAL MYXEDEMA ASSOCIATED WITH GRAVES' DISEASE

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ABSTRACT

In the subject of this case report, hypertrophic osteoarthropathy and pretibial myxedema developed following an induced remission of Graves' disease. Seven similar cases found in the literature are reviewed. It is felt that this association is more than fortuitous and that hypertrophic osteoarthropathy is a rare manifestation of Graves' disease that is probably independent of the hyperthyroid manifestations.

TOXIC diffuse goiter is frequently associated with exophthalmos and ophthalmoplegia and less frequently with pretibial myxedema, a localized collection of mucin in the cutis, usually involving only the lower legs (1). This local form of myxedema occurs only in Graves' disease (1, 2). It may occur during the period of toxicity but more frequently has its onset following control of the hyperthyroidism (1). Patients with pretibial myxedema usually also have exophthalmos.

In addition to these manifestations, the patient to be described presented hypertrophic osteoarthropathy. It is suggested that this is a third sign of Graves' disease that, like the ophthalmopathy and pretibial myxedema, seems to run its course without obvious relationship to the level of thyroid function.

CASE REPORT

Mr. H.R., a Negro 46 years of age, entered Barnes Hospital May 21, 1957 complaining of swelling of his lower legs. He had been perfectly well until the summer of 1952, when he began to suffer from progressive nervousness, sweating, heat intolerance and palpitation. Soon he noted prominence of his right eye. The patient was observed at another university hospital and a clinical diagnosis of hyperthyroidism was made. The thyroidal radioactive iodine uptake was 57 per cent. Following therapy with 12 millicuries of I^{131}, his symptoms improved gradually and he gained weight. Concurrently there was a decrease in the prominence of his right eye.

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In June 1953 he noted slight swelling of his lower legs with irregular thickening and hardening of the skin pretibially and increased local sweating. He again sought medical help at the same university hospital in July 1955. At that time the thyroid was not enlarged. Mild exophthalmos of the right eye and clubbing of the fingers were noted. The skin of the lower legs was markedly thickened. All routine laboratory findings were within normal limits. Studies of thyroid function revealed the basal metabolic rate to be +1 per cent, the serum protein-bound iodine level 6.5 micrograms per 100 ml., the serum cholesterol level 226 mg. per 100 ml., and the thyroidal I^{131} uptake 25 per cent. Radiographs of the hands demonstrated minimal periosteal new bone formation involving several metacarpals and proximal phalanges bilaterally. The skin changes failed to respond to local subcutaneous hyaluronidase or to the delivery of 1500 roentgens to the region of the pituitary.

FIG. 1. Pretibial myxedema, May 1957

In November 1955 he first noted increase in the thickness of the proximal phalanges of his fingers and swelling over the fifth metacarpal bones bilaterally. No discomfort was experienced. He entered Barnes Hospital for evaluation in May 1957. The past history and the review of systems revealed no symptoms referable to the cardiovascular or pulmonary systems. There was no family history of clubbing or arthritis.

On physical examination the patient was found to be a well developed, muscular man who appeared well. The blood pressure was 130/80 mm. Hg, and pulse 74 per minute. The right palpebral fissure was widened. The extraocular movements were normal. The tongue was not enlarged. The thyroid was normal in size, firm and slightly irregular; no bruit was heard. The heart and lungs were normal. No abdominal masses or organs were palpable. The entire skin circumference of each leg from just below the knee to the ankle was markedly thickened and indurated (Fig. 1). There were raised, irregular, nodular areas. The hair follicles were prominent and the individual hairs were coarse and short. The skin was darker than was normal for this patient. Pitting edema (2 plus) was present over the dorsal surface of each foot. The fingers were clubbed (Fig. 2) and the toes were questionably clubbed. Definite bony prominences were palpable over the dorsum of each fifth metacarpal.

Laboratory investigation: The hemoglobin level was 11.9 Gm. per 100 ml. and the white blood count 7,700 per cu. mm., with a normal differential pattern. The basal metabolic rate was −3 per cent and −5 per cent. The thyroidal radioactive iodine uptake was 43.3 per cent in twenty-four hours. The serum protein-bound iodine level was 5.8 μg. per 100 ml. and serum cholesterol level 144 mg. per 100 ml. Radiographic examination of the entire body revealed the following: Periosteal proliferations characteristic of hypertrophic osteopathy were present involving all of the metacarpals and

FIG. 2. Clubbing of fingers, May 1957.

FIG. 3. Hypertrophic osteoarthropathy, May 1957.

proximal phalanges of the hands and the fifth metatarsals (Fig. 3). The degree of proliferation in each instance was obviously greater than in July 1955. There were a few minute periosteal proliferations in several middle phalanges of the hands, presumably similar in nature and etiology. The most minute deviation from the normal appearance was a localized excrescence projecting outward from the diaphyseal cortex; the most advanced proliferations extended the length of the diaphysis but spared the epiphyseal region including the articular cortices. Discrete isolated rarefactions in several of the proliferations of the phalanges were considered to represent preosseous tissue. The cortices of the tibia, fibula and ulna appeared thick but not definitely pathologic. There had been no change in the appearance of these long bones in the interval between July 1955 and May 1957. Microscopic sections from the biopsy specimen of the thickened skin taken by Dr. Zelig Lieberman disclosed large amounts of mucicarmine-positive material deposited between separated collagen fibers. This specimen was typical of pretibial myxedema.

DISCUSSION

Hypertrophic osteoarthropathy has been described as a "chronic proliferative subperiosteal osteitis" (3). In the area of proliferation, new bone is formed which surrounds the original shaft. The tibia, fibula, radius, ulna, metacarpals and metatarsals are most frequently involved. In most cases the osteoarthropathy is associated with clubbing of the fingers and appears to be related to the same process. The cause is unknown. A hereditary form occurs, manifesting itself about the time of puberty. More commonly, however, it appears to be secondary to another disease process. It is especially common in association with disorders of the lung, pleura and mediastinum, but it also occurs with cyanotic congenital heart disease,

cirrhosis of the liver, chronic diarrheas and a large miscellaneous group of diseases (3, 4). In the patient described here, the time of development of the hypertrophic osteoarthropathy and the absence of a known precipitating disorder suggest that it was associated with Graves' disease.

The literature was examined for similar cases. Seven instances including our case are listed in Table 1. Hypertrophic osteoarthropathy was demonstrated radiographically in the 5 cases in which roentgenograms were obtained. Clubbing was present in all cases. The bony changes involved the proximal phalanges and frequently the metacarpals, but involvement of long bone was also reported (5, 6). The onset of thyrotoxicosis was between the ages of 20 and 42. All patients had exophthalmos. The character of the goiter was known only for the subject of this report and for the patient reported by Thomas (5). However, in view of the age incidence and the presence of exophthalmos, it is very probable that all cases fall into the group of diffuse toxic goiter or Graves' disease.

The sex incidence of 6 males to 2 females is in contrast to the usual preponderance of females with Graves' disease but is in keeping with the higher incidence of clubbing in males. Four of the 7 cases were in Negroes.

In all of the cases, clubbing first appeared after successful therapy for hyperthyroidism. The period between therapy and the onset of clubbing

TABLE 1. CLUBBING OF FINGERS IN ASSOCIATION WITH GRAVES' DISEASE

Case report	Patient: Sex & age* (yrs.)	Pretibial myxedema	Therapy for thyrotoxicosis	Time of onset of clubbing after anti-thyroid therapy	Post-treatment hypothyroidism	Roentgenograms
Thomas (5)	M 22	Present	Subtotal thyroidectomy	8 mos.	"Slight"	Hypertrophic osteoarthropathy
Cushing (6)	M 28	? "Large legs"	Subtotal thyroidectomy	8 yrs.	BMR −24%	Hypertrophic osteoarthropathy
Rynearson & Sacava (7)	M 42	Present	Subtotal thyroidectomy	6 wks.	Present	Hypertrophic osteoarthropathy
Engel (8)	F 40	Present	Thyroidectomy	4–11 mos.	Present	Hypertrophic osteoarthropathy
Inch & Rolland (9)	F 36	Present	Subtotal thyroidectomy	Less than 9 mos.	Present	Not reported
Inch & Rolland (9)	M 20	Present	Local radiation	Unknown	Absent	Not reported
Warthin & Boshell (10)	M 23	Present	Subtotal thyroidectomy	Less than 2 yrs.	Absent	Not reported
Present report	M 41	Present	Radioiodine	Less than 10 mos.	Absent	Hypertrophic osteoarthropathy

* At time of onset of thyrotoxicosis.

varied from six weeks to eight years. In 5 of the cases, mild to moderate hypothyroidism was present at the time of the development of clubbing. Thyroid hormone did not benefit the clubbing in any patient to whom it was administered. There is inadequate knowledge for comment on the natural course of the hypertrophic osteoarthropathy. Neither bone pain nor arthralgia has been a prominent feature.

The cause of pretibial myxedema is also unknown. It is said to occur in from 1.6 to 3 per cent of cases of hyperthyroidism (11, 12). Its appearance in 7 and possibly all of the 8 cases listed in Table 1 suggests either that there are common factors underlying hypertrophic osteoarthropathy and pretibial myxedema or that hypertrophic osteoarthropathy is secondary to pretibial myxedema. In 5 of the patients the onset of pretibial myxedema and clubbing were simultaneous. In the others the time of onset of clubbing is unknown. In all of these cases pretibial myxedema, like the clubbing, followed an induced remission of hyperthyroidism. Whether or not these manifestations would have occurred if the hyperthyroidism had remained untreated is a matter for conjecture. However, Ebstein (13) briefly mentions a 24-year-old man with Graves' disease in whom clubbing apparently developed after the onset of toxic symptoms, but before thyroidectomy.

In addition to the foregoing patients, 4 other less well documented cases have been described that may fall into this group. Levitt (14) mentions a patient with Graves' disease, exophthalmos, pretibial myxedema, clubbing and roentgenographic evidence of hypertrophic osteoarthropathy, but he gives no further clinical data. Greene (15) describes a man in whom goiter, exophthalmos and clubbing developed at about the age of 56. Unfortunately there is no information about his metabolic status at that time. Eighteen years later he was found to have progressive exophthalmos, clubbing with hypertrophic osteoarthropathy, and questionable pretibial myxedema. Greene also mentions a young man with exophthalmos and clubbing but without evidence of bony changes and no evidence of preceding thyrotoxicosis. Mendlowitz (16) observed a patient with Graves' disease in whom clubbing developed following thyroidectomy. No further clinical data are given.

It is apparent that hypertrophic osteoarthropathy can occur during the course of Graves' disease. In the cases reviewed, it became evident after induced remission of the hyperthyroid manifestations of the disease; it is usually, or perhaps invariably, associated with pretibial myxedema. Like the ophthalmopathy and the pretibial myxedema, the osteoarthropathy does not seem to depend on the hyperthyroid aspects of Graves' disease.

Acknowledgment

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Addendum

Since the completion of this paper, Freeman (17) has reported 2 cases of Graves' disease and digital clubbing in males. One patient underwent subtotal thyroidectomy and local x-radiation for hyperthyroidism at age 28. At age 56, symptoms and signs of hypothyroidism, exophthalmos and digital clubbing developed simultaneously. There was no pretibial myxedema. Roentgenograms disclosed hypertrophic osteoarthropathy. The other patient, aged 54, is particularly interesting. Digital clubbing developed early in the course of typical Graves' disease, before treatment had been received. Exophthalmos was present, but there was no pretibial myxedema. No roentgenographic studies of the bones were reported.

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Letter to the Editor

EFFECT OF NORETHANDROLONE ON SERUM ENZYME LEVELS*

To the Editor:

A drug reaction characterized by intrahepatic biliary obstruction has been reported in patients treated with norethandrolone (Nilevar) (1–3). Because cellular enzymes appear in the blood in increased amounts in liver disease (4), the effect of norethandrolone on the levels of serum oxalacetic transaminase, serum lactic dehydrogenase and serum aldolase in apparently healthy adults was determined as a control study for a therapeutic drug trial in which these serum enzyme levels are used to assess therapeutic efficacy.

Six laboratory workers were given 30 mg. of norethandrolone daily by mouth for six weeks. Prior to, and at the close of the period of drug administration, serum glutamic-oxalacetic transaminase was determined by the procedure of Karmen *et al.* (5), serum lactic dehydrogenase by the procedure of Meister (6), and serum aldolase by the Dounce *et al.* (7) modification of the method of Sibley and Lehninger (8). Thymol turbidity and procedure of Meister (6), and serum aldolase the Dounce *et al.* (7) modification of the method of Sibley and Lehninger (8). Thymol turbidity and cephalin flocculation were also measured at these times. After blood was drawn for these tests, bromsulfalein excretion was determined.

A consistent increase in all of the serum enzyme levels was observed in all subjects. A marked decrease in bromsulfalein excretion was also found in all subjects. Thymol turbidity and cephalin flocculation tests were not affected by administration of the drug. In general, the subjects with the largest increase in bromsulfalein retention showed the largest increments in serum enzyme levels, and *vice versa*. No subject became jaundiced.

Although the increases in serum enzyme levels are significant, they are not so great as those observed in acute liver disease (4). The increase in lactic dehydrogenase, and particularly in aldolase, an enzyme found primarily in muscle (8), leads to the speculation that the observed increase in serum enzyme levels is due to a direct cellular effect of norethandrolone rather than to secondary hepatocellular involvement as a consequence of biliary obstruction. The marked retention of bromsulfalein in all subjects is consistent with previous reports (1–3). The variation in magnitude of effect from subject to subject with changes of relatively similar degree in

* Supported in part by a grant from the Muscular Dystrophy Association of America, and by a gift from G. D. Searle and Co.

TABLE 1

	Prior to drug*	After norethandrolone, 30 mg. daily for six weeks*	Increment†
Serum transaminase, U./ml.	13.5±2.2 [12–18]	30.5±22.5 [12–69]	17.0 P<0.1
Serum lactic dehydrogenase, U./ml.	50.3±9.1 [36–66]	71.3± 7.0 [66–84]	21.0 P<0.01
Serum aldolase, U./ml.	2.23± .52 [1.8–3.2]	3.77± .77 [2.6–4.8]	1.54 P<0.01
Bromsulfalein retention, %	1.7±1.5 [0.3–4.9]	19.3± 4.0 [12.3–24.3]	17.6 P<0.001

* Mean, standard deviation and ranges of values.
† Mean difference and significance of difference ("*t*" test).

a given subject for the various tests, may indicate a difference in susceptibility to this drug reaction.

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The Endocrine Society

THE 1959 ANNUAL MEETING

The Forty-First Annual Meeting of The Endocrine Society will be held in the Haddon Hall Hotel, Atlantic City, New Jersey, Thursday, Friday, and Saturday, *June 4, 5 and 6, 1959.*

The Chairman of Local Arrangements is Dr. Matthew Molitch.

Scientific Sessions will be held from 9:00 A.M. to 5:00 P.M. daily, and in addition there will be simultaneous afternoon sessions. The annual dinner is scheduled for Friday, June 5, at 7:30 P.M. preceded by cocktails at 6:30 P.M.

All members are urged to make their hotel reservations immediately. The Chalfonte-Haddon Hall Hotel will hold 300 bedrooms for members until May 1, 1959, after which time the hotel will not guarantee further reservations. Therefore it is imperative that you make your reservations now directly with the hotel, advising them of time and date of arrival and departure. Avoid disappointment.

Final program, membership card and advance registration forms will be sent on May 1, 1959 to members whose current dues have been paid.

Those wishing to present papers, which will be strictly limited to ten minutes, should send four copies of the title and abstract to the Vice-President, Dr. Frank Engel, Duke Hospital, Durham, North Carolina, not later than *February 1, 1959. It is imperative that the abstracts be informative and complete with results and conclusions*—not a statement that those will be presented at the meeting—in order that they may be of reference value and suitable for printing in the program. The reading and processing of approximately two hundred abstracts submitted each year and the compiling of the program from these involves a tremendous amount of time and effort on the part of the Program Committee. The Council requests that the authors adhere strictly to the following rules when submitting abstracts, otherwise they cannot be considered:

1. *It is assumed that abstracts submitted for this program have not and will not be submitted elsewhere.*
2. Abstracts may not exceed two hundred words, or equivalent space, exclusive of title. No footnotes or acknowledgments to sponsors can be published. References, if used, must be placed in the body of the

text. The abstract should consist of a single paragraph, if possible. Structural chemical formulas cannot be used.

3. The title heading must be arranged as follows:
   Line 1. Title, not to exceed fifteen words.
   Line 2. Author/s. The name of each nonmember-author collaborating with member-authors is to be followed by the phrase "(by invitation)." Names of the nonmembers who are introduced, *i.e.*, who are not collaborators with member-authors, are to be followed by the phrase "(introduced by . . .)." The principal degree, *e.g.*, M.D., of each author should be written after his name.
   Line 3. Institution of origin and city in which institution is located.
4. The body of the abstract, typed double-space, should follow the heading. The original copy should be on bond paper. There should be three copies.
5. Abstracts should be letter perfect, since there will be no opportunity for proof reading by the authors.

THE 1959 AWARDS

The selection of the recipients of the awards of The Endocrine Society is made by a Committee appointed by Council of the Society. These awards and fellowships carry no obligation by the recipient to the Society or to the donors.

FRED CONRAD KOCH AWARD

During the past year a substantial legacy has been bequeathed to the Society by the late Elizabeth Koch for the purpose of establishing the Fred Conrad Koch Memorial Fund in memory of her late husband, Distinguished Service Professor of Physiological Chemistry at the University of Chicago and pioneer in the isolation of the androgens. This is to be the highest honor of The Endocrine Society and is to be represented by a medal that is to be known as the Koch Medal of The Endocrine Society. The medal, as well as an honorarium of $3,500, is to be given annually to an individual for work of special distinction in endocrinology. The recipient shall be chosen from nominations presented by members of the Society and is limited to citizens of the United States and Canada.

This Award will replace the Medal of The Endocrine Society which was established in 1954 and presented to Dr. Carl R. Moore in 1955, Dr. Frederick L. Hisaw in 1956, Dr. Joseph C. Aub in 1957 and Dr. I. L. Chaikoff in 1958. The Medal of the Endocrine Society replaced the E. R. Squibb Award which was formerly the highest honor bestowed by the Society.

Past recipients of the Squibb Award were Dr. George W. Corner in 1940, Dr. Philip E. Smith in 1941, Dr. Fred C. Koch in 1942, (no award in 1943), Dr. E. A. Doisy in 1944, Dr. E. C. Kendall in 1945, Dr. Carl G. Hartman in 1946, Drs. Carl F. and Gerty T. Cori in 1947, Dr. Fuller Albright in 1948, Dr. Herbert M. Evans in 1949, Dr. C. N. H. Long in 1950, Dr. J B. Collip in 1951, Dr. James H. Means in 1952 and Dr. David Marine in 1953.

THE CIBA AWARD

The Ciba Award, to recognize the meritorious accomplishments of an investigator not more than 35 years of age in the field of clinical or preclinical endocrinology, was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the Award was presented to Dr. E. B. Astwood; 1945—Dr. Jane A. Russell; 1946—Dr. Martin M. Hoffman; 1947—Dr. Choh Hao Li; 1948—Dr. Carl Heller; 1949—Dr. George Sayers; 1950—Dr. Oscar M. Hechter; 1951—Dr. Albert Segaloff; 1952—Dr. Seymour Lieberman; 1953—Dr. Sidney Roberts and Dr. Clara Szego (Mrs. Roberts); 1954—Dr. Isadore M. Rosenberg; 1955—Dr. Jack Gross; 1956—Dr. Alfred M. Bongiovanni; 1957—Dr. Nicholas S. Halmi; 1958—Dr. Monte A. Greer. Prior to 1952 the Award was $1,200. It has now been increased to $2,500.

THE AYERST AND THE SQUIBB FELLOWSHIPS

The Ayerst Fellowship was established in 1947 and the Squibb Fellowship in 1956. They are designed to assist men or women of exceptional promise in furthering their advancement towards a career in endocrinology. Each Fellowship is awarded on alternate years and the stipend, which will not exceed $5,000, may be divided into two Fellowships in varying amounts in accordance with the qualifications of the appointees. Individuals possessing the M.D. or Ph.D. degree, or candidates for either of these degrees, are eligible for appointment.

Applicants must submit the following information:

1. Evidence of scientific ability as attested by studies completed or in progress.
2. Recommendations from individuals familiar with the candidate and his work.
3. A proposed program of study.
4. Acceptance of the individual by the head of the department in which the Fellowship will be held.
5. A statement that he or she will serve full time if awarded a Fellowship. A small amount of time (10 to 15 per cent) may be spent in course work or participation in teaching, the latter purely on a voluntary basis.

THE SCHERING AND THE UPJOHN SCHOLARS OF THE ENDOCRINE SOCIETY

The Council of The Endocrine Society has established a category of Scholars. These grants have been made available through the generosity of the Schering Corporation and the Upjohn Company, and will be awarded to established investigators and teachers in the field of endocrinology who wish to extend their opportunities for work either in this country or abroad.

The awards will not exceed $2,500 annually for each individual and will be granted on the basis of proposals submitted by the applicant. Such applications should include the estimated financial needs. The funds may be used for travel, maintenance and other expenses.

Nominations

Nominations for the Fred Conrad Koch Award, the Ciba Award, and the Ayerst and the Squibb Fellowships may be made by any member of The Endocrine Society. They should be submitted on forms which may be obtained from the Office of the Secretary, 1200 North Walker Street, Oklahoma City 3, Oklahoma. Completed nominations should be returned to the Secretary not later than *October 15* each year.

Proposals for appointments as Scholars of The Endocrine Society should be made in writing by the individual, and addressed to the Secretary of the Society. They should be submitted by *October 15* each year.

The American Goiter Association

THE 1959 ANNUAL MEETING

The next Annual Meeting of The American Goiter Association will be held in the Drake Hotel, Chicago, Illinois, on *April 30, May 1 and 2, 1959.*

THE VAN METER PRIZE AWARD FOR 1959

The American Goiter Association again offers the Van Meter Prize Award of $300.00 and two honorable mentions for the best essays submitted concerning original work on problems related to the thyroid gland. The award will be made at the annual meeting of the Association which will be held in the Drake Hotel, Chicago, Illinois, April 30, May 1 and 2, 1959.

The competing essays may cover either clinical or research investigations, should not exceed 3,000 words in length and must be presented in English. Duplicate typewritten copies, double spaced, should be sent to the Secretary, Dr. John C. McClintock, 149½ Washington Avenue, Albany 10, New York, not later than *January 15, 1959.* The committee who will review the manuscripts is composed of men well qualified to judge the merits of the competing essays.

A place will be reserved on the program of the annual meeting for the presentation of the winning essay by the author if it is possible for him to attend.

JOHN C. MCCLINTOCK, M.D.
Secretary